ROLE OF MAPKs IN ER STRESS-RELATED
CELL CYCLE ARREST AND APOPTOSIS

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ABSTRACT

Endoplasmic reticulum (ER) stress has been implicated in many diseases and cancer therapy. The unfolded protein response (UPR) is activated in the face of ER stress to alleviate it. The survival elements pertaining to the UPR are being investigated for a more effec tive death-induction in cancer cells that have adapted to chronic ER stress and display resistance to treatment. Mitogen-activated protein kinase (MAPK) JNK is implicated in cell pr oliferation, survival and death. JNK is activated during the UPR .

We report in this study that the inhibition of the JNK pathway has different outcomes; depending on the phase of the ER stress response in HCT116 colon carcinoma cells treated with the asparagine-linked glycosylation inhibitor tunicamycin (TM). Both outcomes indi cate a cytoprotective role for JNK activity during ER stress -related cell survival and death. TM treatment at 1 µg/ml induced cell cycle arrest at the G1 phase. Cell cycle arrest can be interpreted as a cytoprotective response that conserves energy as an a daptation strategy to ER stress. Inhibition of JNK by the small molecule SP600125 led cells bypass this arrest compared to TM-treated HCT116 cells. Cell proliferation in response to JNK -inhibition was observed by light microscopy. Accordingly, p21CİP1 pro tein, a cyclin-dependent kinase inhibitor (CKI) that causes cell cycle arrest at the G1 to S transition was elevated in TM -treated cells, whereas JNK inhibition abrogated this upregulation. It has been reported that JNK activity stabilizes p21CİP1, which h as short half-life of 20 – 60 min.

TM-induced apoptosis involved caspase-3 and -8 activation and cells were partially rescued from death by the administration of the pan-caspase inhibitor z-VAD-fmk. Initiation of apoptosis coincided with the increase in p21CİP1 levels in JNK-inhibited TM-treated cells after 24 h of treatment. JNK inhibition caused a 2-fold increase in apoptosis in TM-treated cells after 24 h, after which the effect of JNK inhibition did not increase cell death. Inhibition of the MAPKs p38 and ERK did not effect the cell cycle distribution of TM-treated cells and there was no change in the apoptotic response after 24 h of treatment. p38 and ERK inhibition sensitized HCT116 cells to ER stress-related apoptosis after 40 h of treatment. In this experimental model, JNK and p38 and ERK differed temporally in their prosurvival roles, presumably due to the condition of the cell and the availability of affected substrates.

ER STRESLE İLGİLİ HÜCRE DÖNGÜSÜNDEN KAÇIŞ VE APOPTOZDA MAPKlerin ROLÜ

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ÖZET

Endoplasmik retikulum (ER) stres birçok hastalık ve kanser tedavisiyle ilgildir. ER stresli hücrelerde bu durumu gidermek ve homeostaz koşullarına geri dönmek için "Katlanmamış Protein Cevabı" mekanizması aktive olur. Kronik ER strese "Katlanmamış Protein Cevabı" aktivasyonu ile adapte olmuş ve tedaviye cevap vermeyen kanser hücrelerinde daha etkili bir sonuç verecek ilaç uygulaması için "Katlanmamış Protein Cevabı"nda hücreyi hayatta tutan öğeler araştırılmaktadır. MAP kinazlardan JNK, hücre çoğalması, hayatta kalması ve ölümle ilişkili bir proteindir. JNK "Katlanmamış Protein Cevabı" sürecinde aktive olur.

Bu çalışmada tunikamisin (TM) ile ER stres kondisyonu yaratılmış HCT116 kolon karsi noma hücrelerinde JNK inhibisyonunun ER stres cevabının farklı fazlarında, farklı sonuçlar yarattığını gördük. Bu iki tip netice de ER stres sırasında JNK aktivitesine sitoprotektif bir görev atamaktadır. 1 µg/ml TM uygulaması hücrelerin hücre döngüsünün G1 fazında kalmasına yol açtı. Hücre döngüsünden bu kaçış sitoprotektif bir cevap olarak düşünülebilir çünkü hücre bölünmesini engelleyen bu döngüden kaçış hücreye enerji tasarruf ettirir ve ER strese adaptasyonu kolaylaştırır. SP600125 isimli ufak molekülle JNK'ın inhibe edilmesi, bu hücrelerde hücre döngüsünün devam etmesine yol açtı. Ayrıca bahsi geçen JNK inhibe edilmiş ER stresli hücrelerde hücre çoğalması optik mikroskop altında takip edildiğinde hücrelerin çoğalmaya devam ettiği gözlenmiştir. Buna paralel olarak, p21CİP1, G1 fazından S

fazına geçişi engelleyen siklin-bağlı kinaz engelleyici protein miktarı TM uygulanmış hücrelerde artış gösterirken, JNK inhibisyonu bu artışı engellemiştir. Daha önceki çalışmalardan JNK aktivitesinin, yarı ömrü 20 - 60 dk olan p21CİP proteinini stabilize ettiği bilinmektedir.

TM uygulanan hücrelerde gözlenen apoptozda kaspaz-3 ve -8 rol oynamıştır. Bu hücrelere genel-kaspaz inhibitorü olan z-VAD-fmk uygulandığında hücreler apoptozdan kısmen kurtulmuştur. TM uygulanmış ve JNK inhibe edilmiş hücrelerde apoptoz başlangıcı p21CİP1 proteininin artışıyla paralellik göstermiştir. JNK inhibisyonu 24. saatin sonunda bu hücrelerde apoptozu, sadece TM uygulanmış hücrelere göre iki kat artırmıştır. 40. saatte bakıldığında ise sadece TM uygulanmış hücreler ile hem TM uygulanmış hem de JNK inhibe edilmiş hücrelerin apoptoz oranlarında bir fark gözlenmemiştir. TM uygulanmış hücrelerde, p38 veya ERK MAP kinazlarının inhibisyonu hücre döngüsü dağılımını değiştirmedi. Ayrıca 24. saatte bu hücrelerin apoptotik tepkisinde TM uygulanmış hücrelere göre bir farklılık gözlenmemiştir. Fakat 40. saatte p38 veya ERK inhibe edilmiş hücrelerde apoptoza duyarlılık gelişmiştir. Bu durumda deneysel modelimizde, JNK ve ayrı bir grup olarak p38 ve ERK kinazlarının ER stresli hücrelerdeki hayatta kalmayı destekleyici rollerinde zamansal farklılık gözlenmiştir. Bunun olası sebebi hücrenin durumu ve hücre içinde bu proteinlerin (ERK, p38, JNK) etkileyebileceği substratların mevcudiyeti olabilir.

"To the liberation of women"

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

ER	Endoplasmic reticulum
UPR	Unfolded protein response
TM	Tunicamcyin
ERAD	ER-associated protein degredation
BiP/GRP78	Immunoglobulin heavy chain-binding protein/Glucose-regulated protein of mw 78
PERK	Pancreatic ER kinase (PKR)-like ER kinase
ATF6	Activating transcription factor 6
IRE1	Inositol-requiring enzyme 1
ERSE	ER stress response element
UPRE	Unfolded protein response element
PARP	Poly ADP Ribose
MOM	Mitochondrial outer membrane
MOMP	Mitochondrial outer membrane permabilization
IAP	Inhibitor of apoptosis
PS	Phosphatidylserine
MAPK	Mitogen-activated protein kinase
ASK1	Apoptosis signal-regulating kinase 1
MAPKKK	MAP kinase kinase kinase
MAPKK	MAP kinase kinase
JNK	c-Jun N-terminal kinase
ERK	Extracellular signal-regulated kinase
PI3K	Phosphatidylinositol 3 kinase
CDK	Cyclin-dependent kinase
CKI	Cyclin-dependent kinase inhibitor
eIF2α	Elongation initiation factor 2 alpha
HCT116	Human colon carcinoma cell line

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INTRODUCTION

1.1 The Endoplasmic Reticulum

The endoplasmic reticulum is a membrane-bound labyrinth-like cellular compartment in the cytoplasm of eukaryotic cells. It is an organelle essential for the translation, maturation, modification, and delivery of nascent polypeptides to be secreted outside of the cell or that are destined to function at the plasma membrane and other intracellular organelles. All secretory proteins traverse the ER in order to enter the secretory pathway [1], making up approximately one third of the proteins synthesized in the cell [2].

Here, polypeptide chains fold into their native states in a chaperone-mediated process. Chaperones use the energy from ATP hydrolysis to promote folding and prevent aggregation of proteins within the ER [2]. Nascent proteins get subjected to disulfide bond formation catalyzed by disulfide isomerases [3].The oxidizing environment of the ER is necessary for the formation of disulfide bonds and the proper folding of nascent polypeptide chains [4].

Asparagine-linked glycosylation of proteins takes place in the ER [5]. Glucose not only provides the metabolic energy for protein folding and modification but directly participates in glycoprotein folding, as a component of the oligosaccharide structures added to consensus asparagine residues of the maturing glycosylated proteins in their polypeptide chain state. With the addition of the oligosaccharide core, the three terminal glucose residues are cleaved by glucosidases. The ER administers tight quality-control systems for the recognition of unfolded/misfolded proteins. In the case of glycoslyation, UDP-glucose:glycoprotein glucosyltransferase (UGGT) recognizes the misfolded protein and reglycosylates the core oligosaccharide structure. The reestablishment of the glucose-α(1,3)-mannose glycosidic linkage in this manner ensures the retaining of the unfolded protein within the ER lumen by promoting its binding to ER-resident protein chaperones calnexin and calreticulin [6].

The ER has other quality-control mechanisms, which collectively assure that only those proteins that are properly folded leave the organelle. Proteins are kept in the lumen until they reach their native conformation or are translocated to the cytosol for their degradation by the 26S proteasome [7].

The ER, apart from this prodigious task of protein folding is the primary storage organelle for cellular Ca^{2+}. The high concentration of Ca^{2+} is retained in the ER through the activity of Ca^{2+} ATPases. Molecular chaperones and foldases that are necessary for protein folding require Ca^{2+} to aptly function [8].

1.2 Homeostasis at the ER and its Antagonist, the Unfolded Protein Response (UPR)

Proper functioning of the ER is crucial for a healthy operating cell. The ER lumen maintains an extremely high concentration of proteins, estimated at 100mg/mls [9]. Protein synthesis rate in the ER for secretory cells is especially high ranging between approximately 2.6 million secretory proteins for hepatocytes to 13 million for exocrine cells per minute. The translation and modification of copious amounts of proteins at the ER is thermodynamically unfavorable; therefore, a large amount of energy is required to accomplish the task [10]. The ER homeostasis is prone to many insults due to this colossal undertaking. The ability of the cell to sense, respond and overcome the stress is essential for maintaining homeostasis and remaining viable. Highly specific signaling pathways ensure that the ER does not lose its homeostatic balance and that its protein-folding capacity is not inundated.

The pathways activated to keep the ER at homeostasis against certain stress conditions are collectively named the unfolded protein response (UPR). The stress that activates the UPR can be due to many different factors related to the proper working conditions and undertakings of the ER, such as perturbations in calcium concentration and redox status, increased protein synthesis, expression of mutated proteins, impairment of protein transport to the Golgi, glucose deprivation, and unsuccessful glycosylation [11]. ER stress brings about the accumulation of misfolded/unfolded proteins in the ER lumen, which activates the UPR. During the UPR certain evolutionarily conserved cellular pathways are activated resulting in a decrease in ER's protein load, an increase of its folding capacity and the degredation of misfolded proteins. The measures are taken to bring back the conditions of homestasis to the ER. There are conditions where the UPR works as an adaptive mechanism to chronic stress and is always active to maintain cellular balance. The outcome of persistent ER stress may be the death of the cell in a way related to UPR activity.

1.2.1 ER stress in the sick and healthy body

There are physiological conditions of the body, in which ER protein folding capacity is exceeded. The outcome of the stress may be acute (transient), with recovery on the order of minutes to hours or chronic (persistent). Acute stress is relieved best by an immediate reduction in the protein load of the ER. On the other hand, chronic stress requires long term adjustments to cellular function. Chronic ER stress may be due to genetic, environmental, pathogenic or developmental factors [12].

In development, the differentiation of B lymphocytes into antibody-secreting plasma cells results in massive ER expansion. ER chaperones in these cells are upregulated as a consequence of UPR activation [13]. Active UPR maintains homeostasis in these cells upon an increase in secretory protein production. Other examples to chronic stress are mutations in genes encoding secretory or transmembrane proteins, such as α1 antitrypsin and insulin, and viral infections such as hepatitis C [14]. It is to note that in the case of adaptive UPR, it would be interesting to understand the factors that lead cells bypass the potentiality of demise and adapt to chronic ER stress.

Hypoxia is a common feature of malignant and resistant solid tumors resulting from deregulated growth and vascular abnormalities associated with the tissue. Hypoxia in cells activates the UPR to adapt to the low-oxygenated environment [15].

Conformational diseases are diseases caused by the misfolding and aggregation of proteins [16]. The accumulated unfolded or misfolded proteins in the ER form aggregates in the ER lumen and the cytosol [17]. The cause of many neurodegenerative diseases is the death of cells with accumulated unfolded proteins, as an outcome of UPR.

UPR is also activated in cells faced with altered metabolic conditions, such as glucose deprivation, hyperhomocysteinemia and ischemia [15].

UPR activity has also been detected in cells that are considered unstressed. The basal UPR activity was implicated in nutrient sensing and control of cellular responses to fluctuations in nutrient levels. This extends the physiological functions of the UPR [1].

1.2.2 Induction of ER stress by Pharmacological Agents

Commercial pharmacological agents that mimic ER stress are available for experimental use [16]. These pharmacological agents inhibit certain functions of the ER that are essential for proper protein maturation.

One group of pharmacological ER stressors comprises asparagine-linked glycolysation inhibitors that disrupt asparagine-linked glycolysation of proteins at the ER lumen. 2-Deoxy-D-glucose and tunicamycin (TM) [18] are of this kind.

Another group of ER stressors disrupts the Ca^{2+} metabolism at the ER. ER lumen requires high concentrations of Ca^{2+} for the ER chaperones (such as GRP74/BiP) and foldases to properly function. Ca^{2+} ionophores such as A23187 and the Ca^{2+} pump inhibitor, thapsigargin (TG) elicit ER stress.

A third group comprises reducing agents disrupting the oxidative environment of the ER lumen that is required for the formation of disulfide bonds between the cysteine residues of nascent peptides for folding into their tertiary and quaternary structures. Dithiothreitol and 2-mercaptoethanol are of this kind of pharmacological ER stressors.

Much of the knowledge about the UPR, its activation and regulation, comes from cultured cells treated with these ER stress-inducing agents. It should be noted that this kind of experimentation bares a shortfall in its nature that it fails to imitate the UPR under milder and persistent assault, which is often the case in the physiological condition. There are cases, where cells, when faced with chronic stress activate the UPR in an adaptive form and escape death in a scale ranging from days to years. Treatment with these agents severely assaults the cells under investigation and eventually activates the death mechanism linked with the UPR. In vitro experimentation, therefore, may not be the equivalent of the physiological UPR or more precisely, the problem with this methodology is that it circumvents the question of how cells escape death and adapt to ER stress [15].

1.2.2.1 Tunicamycin

Tunicamycin is an antibiotic produced by *Streptomyces lysosuperificus*. Tunicamycin (TM) induces ER stress by inhibiting N-linked glycosylation of proteins in the ER lumen by preventing UDP-GlcNAc-dolichol phosphate GlcNAc-phosphate transferase activity [18]. Tunicamycin activates the ER stress response in a variety of cell types.
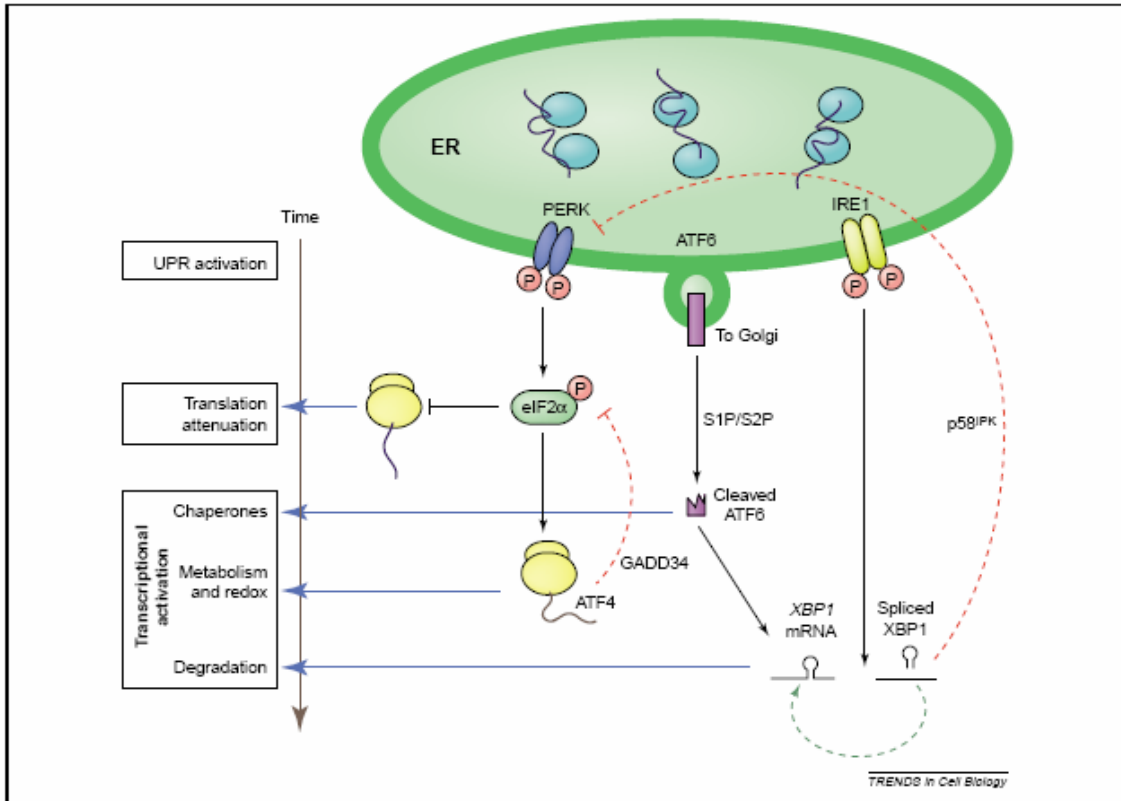
Figure 1.1 [19] The basic pathways of the UPR mediated by the three ER-transmembrane proteins functioning as ER-stress sensors.

Each sensor protein activates a pathway in a time-phase dependent fashion. An example pertaining to the time-phase characteristic of the response is that for the full-fledged activation of the IRE1 pathway, the transcription factor ATF6 must be processed (cleaved) to produce the precursor XBP1 mRNA that is a substrate of IRE1's. This makes sure that the downstream response of ATF6 is induced earlier than the downstream response of IRE1-XBP1. Positive and negative feedback loops within the same or different pathways ensures continious or transient response patterns within cross-talking pathways. The green and red dashed lines represent the positive, and negative feedback loops, respectively. The specific UPR pathways are not mutually exclusive as depicted in the figure in terms of gene activation. Some genes require the concurrent activaton of duplicate or multiple sensing pathways.

1.3 A Closer Look at the UPR Execution Pathways

As a summary: ER stress results in the accumulation and aggregation of misfolded proteins in the ER lumen. The cell responds to it by activating precise and evolutionarily conserved intracellular mechanisms in order to bring the ER back to the homeostatic conditions. This fundamentally cytoprotective response is called the unfolded protein response (UPR). **Figure 1** shows the basics of the UPR mechanism that is maintained upon the accumulation of misfolded proteins. Distinct components (pathways) of the UPR depicted in the figure will be covered in terms of protein activity, gene regulation and their consequences with an emphasis on the time shift property of the progress of the response.

The cell employs the activation of the UPR subpathways in a time-dependent fashion [20]. The precedent response comprises the blockage of global protein translation. Translational attenuation serves a brilliant role in reducing the protein load of the semi-functional ER, thus diminishing damage to the organelle. Next is the increase in the folding capacity of the ER through upregulation of ER-resident molecular chaperones [21], foldases (enzymes including protein disulfide isomerase (PDI) and peptidyl-prolyl isomerase) and increase in the size of the ER [22].

What comes after the response to augment of ER folding capacity in the "time-dependent phase-shift response" is the ER-associated protein degradation (ERAD) [20] pathway is the primary degradation mechanism for handling misfolded proteins that cause the stress response [23]. ERAD includes the transcriptional induction of ER degradation-enhancing α-mannosidase-like protein (EDEM) to eliminate the misfolded proteins in the ER via the 26S proteasome [24]. EDEM is critically involved in the quality control of proteins in the ER by targeting misfolded glycoproteins to ERAD.

If the stress persists despite of the activated stress response, apoptotic machinery takes over to eliminate the ER stress-damaged cells [25].

For the cellular machinery to respond to ER stress and activate the UPR; however, it first and foremost must sense it. Sensing the stress is the responsibility of ER-resident chaperones and the three ER transmembrane proteins, PERK, ATF6 and IRE1.

1.3.1 ER-resident Chaperone BiP Interacts with the ER-transmembrane Proteins

To form secondary and tertiary structural elements in which residues far apart in the amino acid sequence interact, preceding residues mu st be kept in a folding-competent state while the polypeptide chain grows. In the ER, ER-resident chaperones takeover the task of proper protein folding. Bip/GRP78 (immunoglobulin heavy chain-binding protein/glucose-regulated protein of molecular weight 78 kDa) is the ubiquitously expressed chaperone in the ER. BiP belongs to the heat-shock protein (Hsp70) family. It contains an ATPase domain at its N-terminus and a substrate binding domain at its C-terminus, binding to the hydrophobic residues of folding intermediates. In its ADP-bound form, it has high affinity for its protein substrates. BiP binding to its substrate stimulate s its ATPase activity. Exchange of ADP with ATP brings BiP to its low affinity state for its substrates [26]. Subsequent ATP hydrolysis returns BiP into its ADP-bound form. Folding polypeptide chain thus requires and consumes ATP. Co-chaperones are in action for nucleotide exchange and ATP-hydrolysis. Conformations that interact with molecular chaperones are considered unfolded.

The regulatory signaling proteins at the ER membrane that respond to the accumulation of unfolded or misfolded proteins in the ER lumen act through an interaction with BiP. These transmembrane proteins initiate the stress response signaling [2] that will either result in the restoration of ER homeostasis or death of the cell [27].

In mammals, the three ER transmembrane proteins that sense and mediate the UPR are the pancreatic ER kinase (PKR)-like ER kinase (PERK), activating transcription factor (ATF6), and inositol-requiring enzyme 1 (IRE1) **(Figure 2)**. These sensor proteins associate with BiP under non-stress conditions at their ER-lumenal domains. The hypothesis is that a s unfolded proteins accumulate in the ER upon stress, BiP dissociates from these UPR transducers and preferentially binds to the unfolded/misfolded proteins [28].

BiP associates with the lumenal domain of IRE1α, possibly at multiple sites with a hydrophobic character. The domains required for signaling, oligomerization and BiP-binding of IRE1α partially overlap. Therefore, an assumption is that BiP-binding inhibits IRE1α oligomerzation to keep it in its inactive state [29].

The lumenal domain of PERK is interchangeable with IRE1's . It is highly probable that BiP binds to PERK's luminal domain in a similar fashion. However, the oligomerization and BiP-binding domain of PERK are distinct. Therefore, in this case, BiP might interfere with PERK oligomerization through inducing a conformational change in PERK's oligomerization domain [30].

In ATF6, BiP binds to one (GLS1) of the two independent and redundant Golgi localization sequences (GLS1 and GLS2) of ATF6. In the absence of BiP binding, GLS2 results in the translocation of ATF6 to the Golgi and causes its subsequent activation [31].

The three sensors of the UPR are activated simultaneously under severe ER stress conditions. However, under various physiological conditions, selective UPR subpathways are activated. One example is that, the activation of B-cell differentiation in response to the UPR results in the activation of only the IRE1 pathway [13].

The pathways activated by the three sensors are distinct and these three UPR subpathways require unique and important lag times before they are full-fledged. The most rapid response is the result of PERK activation.
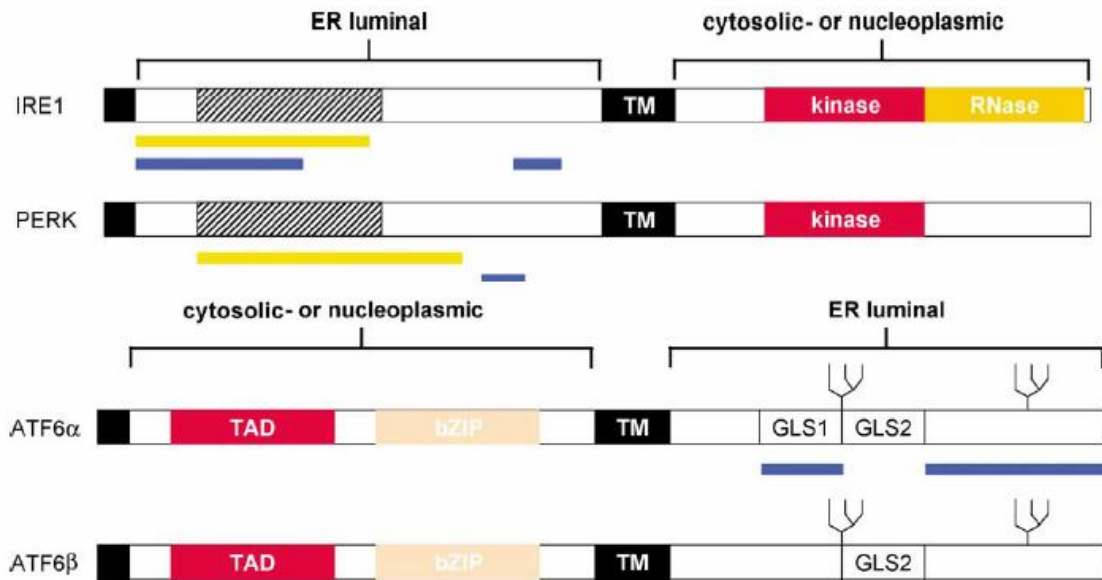
Figure 1.2 [32]. The three ER-stress sensors, IRE1, PERK and ATF6.

Orange bars are the regions for signal transduction and oligomerization. Blue bars are the regions for BiP binding. The black box at the terminal domain is the signal peptide. Hatched boxes are the regions of limited homology between IRE1 and PERK. IRE1 and PERK oligomerization domains partially overlap.

Abbreviations are: bZIP – basic leucine zipper, GLS1 and GLS2 – Golgi localization sequences 1 and 2, TAD – transcriptional activation domain, TM – transmembrane domain. Note: Drawings are not to scale.

1.3.2 PERK

PERK is an ER-resident type I transmembrane serine/threonine protein kinase [22]. Upon its release from BiP, PERK oligomerizes, autophosphorylates and becomes an active kinase [28]. PERK, once activated, phosphorylates the Ser51 residue of eIF2α. [22] causing the attenuation of global protein translation. The activity of PERK in protein translation attenuation is a key element in the survival mechanism triggered by the UPR. Mouse embryonic fibroblasts (MEF) with a homozygous mutation on eIF2α at Ser51 are sensitive to ER stress [33]. *perk-/-* cells can be partially rescued from early death by translation inhibitors, such as cycloheximide, when subjected to ER stress [34].

The PERK arm of the UPR is activated most rapidly because its substrate eIF2α is a direct substrate of PERK. PERK, once activated phosphorylates eIF2α without a requirement for

nuclear translocation or translation or transcription. This rapid response prevents further influx of nascent proteins into the ER.

Phosphorylation of eIF2α attenuates the general translation rate, while it allows for preferential translation of mRNAs encoding upstream open reading frames (uORFs) **(Figure 3).** The transcription factor ATF4 is one example. Proteins carrying a uORF like ATF4 are transcribed more efficiently when eIF2α is inactive due to phosphorylation [35].

ATF4 mRNA is found at the cytoplasm and is rapidly translated upon this global translational attenuation. The notable target genes of the ATF4 transcription factor are CHOP/GADD153 and GADD34 [35], [36]. ATF4 is also responsible for activating genes that are involved in amino acid import, glutathione biosynthesis and resistance to oxidative stress [34].

The activation of HSP40 co-chaperone p58^{IPK} by ATF6 and GADD34 later in the response is a negative feedback mechanism to reestablish global translation through dephosphorylation of eIF2α. GADD34 and p58^{IPK} expression is induced later in the ER stress response [37].

In the case of translational attenuation, short-lived proteins are eliminated from the cell. Cyclin D1 is one noteworthy example. The loss of cyclin D1 results in the arrest of mammalian cells in the G1 phase of the cell cycle [38]. The significance of cyclin D1 loss will be discussed in more detail in Chapter 1.5.
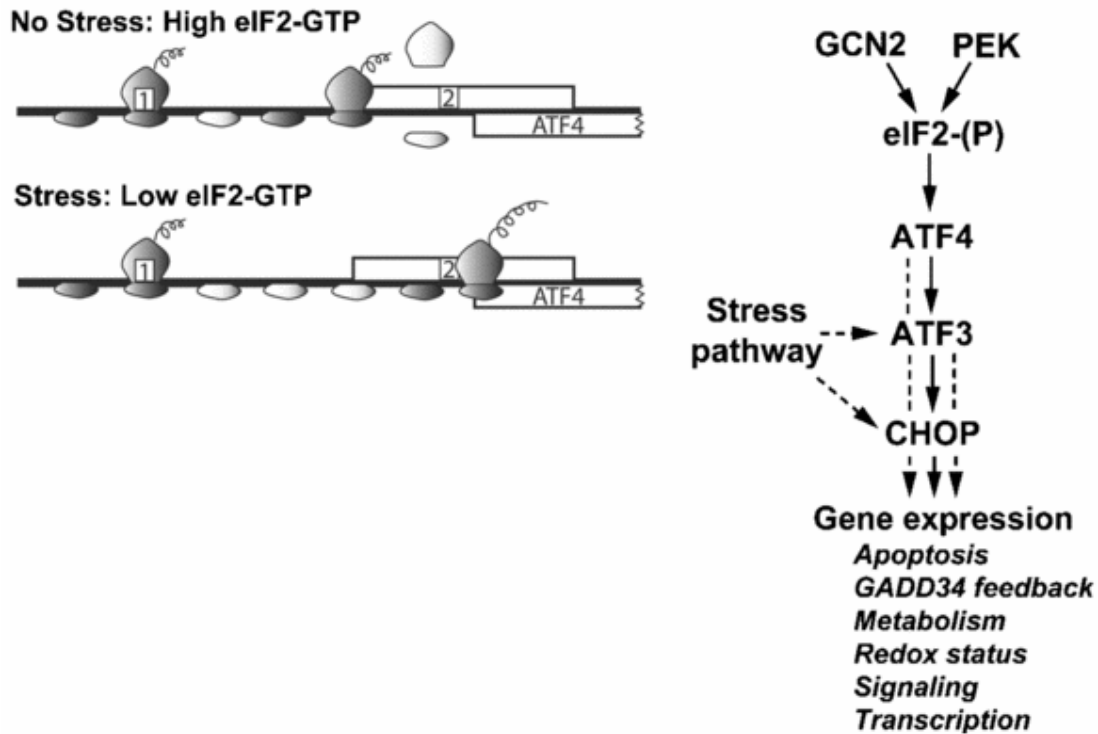
Figure 1.3 [39] Mechanism of eIF2α phosphorylation during stress

When eIF2-GTP level is high protein translation continues. eIF2α phosphorylation by PERK during the UPR leads to low levels of eIF2-GTP complex, causing attenuation of global protein translation, while bringing about an increase in the translation of mRNAs with upstream open reading frames (uORFs). The ATF4 mRNA is depicted as a line with the coding region as a box. The uORFs are numbered as 1 and 2. During stresss, low eIF2 -GTP level facilitates the bypass of the inhibitory uORF2 enhancing the translation of ATF4 mRNA. The targets of the transcription factor ATF4 are genes that regulate stress response such as ATF3 and CHOP. In addition, the activity of ATF3 and CHOP can be regulated by the mitogen-activated protein kinase (MAPK) pathways.

1.3.3 ATF6

ATF6 is a type II ER transmembrane protein with two forms, ATFα and ATF6β. With the activation of the UPR, ATF6 translocates to the Golgi from the ER membrane [28], where it is cleaved by site-1 and site-2 proteases (S1P and S2P) [40].

Upon cleavage, ATF6α and ATF6β, comprised of an N-terminal cytosolic domain containing the bZIP transcription factor motif translocate to the nucleus, where they bind the *cis*-acting ER stress response element (ERSE), ERSE-I and ERSE-II consensus sequences 5'-CCAAT-N_9-CCACG-3' and 5'-ATTGG-N-CCACG-3', respectively as a homo- or a heterodimer.

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Transcription factor NF-Y is required for their binding to ERSE-I [41]. The ERSE consensus sequences are necessary for the induction of the GRP genes [42]. Using the ERSE sequence as a probe, Yoshida et al. cloned the transcription factor ATF6.

Important target genes of ATF6 are BiP, XBP-1, CHOP [43], and p58^{IPK} [44].

1.3.4 IRE1α and IRE1β

IRE1 is a type I ER transmembrane protein with a cytosolic serine/threonine kinase and a site-specific endoribonuclease domain and a lumenal (ER) dimerization domain [45], [46]. IRE1α and IRE1β have been identified as the two mammalian homologs of yeast IRE1p that releases stress signals from the ER resulting in the activation of the UPR [45]. IRE1 arm of the UPR is the only arm yeast possesses. The IRE1 pathway is the oldest pathway since it is conserved throughout the eukaryotes.

The two mammalian forms IRE1α and IRE1β are expressed ubiquitously in all cells and specially in intestinal epithelial cells, respectively [47].

IRE1 is activated by oligomerization and autophosphorylation via its kinase domain when the ER lumen chaperone BiP/GRP98 dissociates from its luminal domain. The RNase domain of IRE1 serves to cleave a 26-nucleotide intron of XBP1 mRNA to cause a translational frameshift that yields XBP-1's bZIP transcription factor activity once translated [48].

As mentioned above, target genes of the UPR share specific consensus sequences in the ir gene promoters: the *cis*-acting ER stress response element (ERSE) [42]. Apart from the ERSE sequence, XBP-1 binds to the unfolded protein response element (UPRE) with the transcription factor NF-Y [11]. Both ERSE and UPRE promoters are activated by bZIP-containing transcription factors.

XBP1-mediated response provides both the transcription of the ER chaperone genes via ERSE-mediated transcription and also EDEM via UPRE-mediated transcription to degrade unfolded proteins through ERAD [20]. EDEM is an ER-resident type II transmembrane protein that binds to misfolded glycoproteins to enhance their degradation [49]. The ERAD

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mechanism represents the third step of the UPR, along with attenuation of translation and expansion of the ER folding capacity.

Response of the IRE1 arm of the UPR, thus the activation of ERAD, is slower compared to the other subpathways because of the fact that XBP1 mRNA is expressed in low levels in nonstressed cells. Expression of the XBP1 mRNA is upregulated by ATF6 once the response initiates [48]. However, once it is activated, XBP1 activation is more sustained than ATF6's because XBP1 carries an ERSE sequence in its promoter region to transactivate its own transcription with positive feedback unlike ATF6.

One point about the common features of the three arms of the UPR is that they directly or indirectly activate a complex network of bZIP transcription factors. Another level of control in the ER stress response comes from the engagement of bZIP proteins in crosstalk and activity regulation through homo and/or heterodimerization [32].

1.3.5 Markers of the UPR

The characterization of the mammalian unfolded protein response is realized by the transcriptional increase in genes that encode the ER molecular chaperones, such as BiP/GRP78 and a non-chaperone, C/EBP homologus protein CHOP/GADD153. Increase in the mRNA or protein levels of Bip/GRP78 and CHOP are typically used as markers of the UPR [50].

1.4 Prolonged ER Stress Leads to Apoptosis

If the UPR cannot overcome the stress, or if the stress is prolonged, signaling for the demise of the cell takes over [27]. ER stress not only activates the apoptosis machinery but another death-related mechanism as well, which is autophagy. Next section will primarily deal with the activaton of apoptosis machinery as a result of stressed ER.

1.4.1 Apoptosis

The basic tenets of the apoptosis pathway are conserved in all metazoans. Its operation is vital to embryonic development in terms of organogenesis and the formation of tissues. Controlling the death of cells along with division seems necessary for the vitality of a developing embryo. Apoptosis also operates in adult organisms and its malfunction causes serious problems. Insufficient apoptosis in an adult organism manifests itself as cancer or autoimmunity, whereas excess apoptosis results in degenerative diseases, immunodeficiency and infertility [51].

Studies in apoptosis took an edge with the start of use of *C.elegans* as a model organism. In *C.elegans*, the same 131 cells out of 1090 die during development. The cloning of genes responsible for the death of these cells marked the boosting of the field. By mutagenizing *C-elegans*, the genes ced-3, ced-4 were found to be absolutely required for cell death. ced -9, the worm homolog of the mammalian Bcl-2 oncogene, was first identified as preventing cell death. The findings brought Brenner, Horvitz and Sulston the Nobel Prize in 2002.

ced-3 or ICE (interleukin 1 beta converting enzyme) became the first member of the caspase family, caspase-1. Caspases are a family of proteases dependent on their cysteine residues to cleave aspartic acid motifs. Caspases are inactive zymogens consisting of a large and small subunit preceeded by an N-terminal prodomain before they are cleaved to function as active proteases.

Apoptosis is considered as Type I programmed cell death. Type II cell death requires the participation of lysosomes. Distinction of death types is discussed in the Extras chapter, with an emphasis on activated autophagy in response to stress signaling. Programmed cell death was first coined to define the process of the consistent changes of a failing cell with the possibility of experimentally preventing this phenomenon [52]. Today, it means that cells possess genes that can bring about their own destruction and that almost all physiological and most pathological cell deaths are managed, rather than disordered [52].

Apoptotic cell death depends on the breakdown of the cell by the eventually activated caspases. Caspases can be activated through two distinct apoptotic pathways. One pathway fires out with the oligomerization of cell surface death receptors in response to the binding of

their corresponding ligand at the extracellular space. This pathway is denoted as the extrinsic apoptotic pathway of apoptosis. The intrinsic pathway involves the mitochondria and cytochrome c release from it to form the apoptosome to a ctivate the executioner caspases.

The hallmarks of apoptotsis are membrane blebbing, chromatin condensation, nuclear fragmentation, DNA degradation into a distinct ladder form and cleavage of cellular proteins, such as protein kinase C-δ (PKC-δ) and PARP [53], [54].

There are critical proteins in the apoptotic pathway that regulate the life and death decisions of the cell. Bcl-2 family member proteins are of great importance.
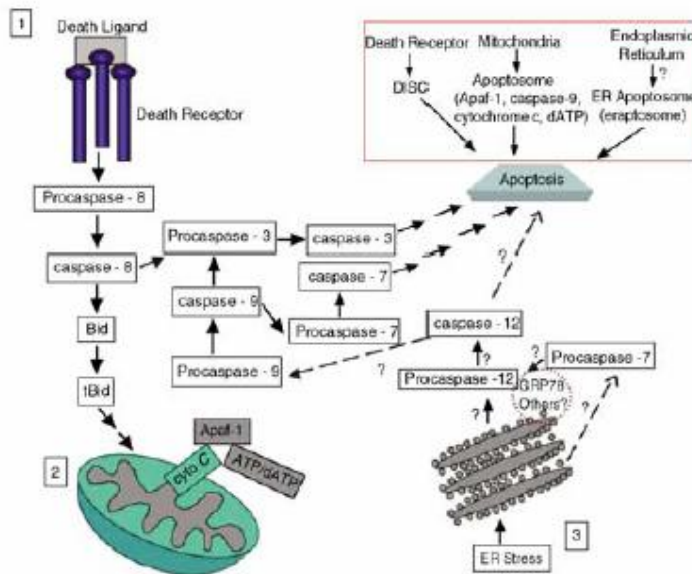
Figure 1.4 [55] The caspase cascade of the extracellular and intracellular apoptosis pathways.

Extracellular apoptosis pathway merges with the mitochondrial pathway in many cases, most likely through BH3-only members of the Bcl-2 protein family. In this figure, signals from the stressed ER constitute the third pathway of apoptosis. In rodents, caspase-12 is activated in response to ER stress. A homolog of caspase-12 is non-functional in humans. Active caspase-12 activates caspase-9 for the execution of apoptosis. The solid arrows are the known pathways, question marks are unconfirmed interactions and pathways.

1.4.1.1 Bcl-2 Family Proteins and the Intrinsic (Mitochondrial) Apoptosis Pathway

Members of the Bcl-2 family have distinct response patterns to specific death stimuli. They have specific subcellular localizations and developmental expression [56].

Mammals possess the entire family of Bcl-2 proteins that include proapoptotic and prosurvival members. The first Bcl-2 family member protein identified is Bcl-2. The first proapoptotic member is Bax, which was identified by its interaction with Bcl-2 [57]. There is a delicate balance of proapoptotic and prosurvival factors in the cells to keep them in viable [58]. The ratio of proapoptotic and prosurvival molecules such as Bcl-2 and Bax constitutes a threshold for susceptibility of the cell to die. However, the mechanism of keeping this balance is far from being direct. There are many factors and pathways involved.

A noteworthy reminder is that it is good to keep in mind that molecules that play a role in the life-death decision of the cell are tended to be attributed prosurvival or prodeath depending on how they affect the fate of the cell in controlled experimental setups. Bcl-2 is one of the proteins that is labeled as prosurvival. However, recent evidence suggests that in certain conditions, abiding by the dichotomy of prosurvival/death classification, Bcl-2 acts as a prodeath protein depending on its subcellular localization. There are other examples of similar paradoxical observations. However, I will mention about these proteins adhering to their generic attribution unless I speak of their non-canonical ways of functioning.

Bcl-2 family proteins are generally divided into three types based on their homology of the possible four major domains (BH1 – BH4). This classification also corresponds to their role in the death process, prosurvival or proapoptotic. Prosurvival Bcl-2 family proteins possess all four BH domains. Bcl-2, Bcl-XL, Mcl-1 belong this group. The crystal structure of the Bcl-XL monomer reveals that its BH1, BH2 and BH3 domains are in close proximity to create a hydrophobic pocket which can bind the BH3 domain of proapoptotic members of the family [59].

Proapoptotic members, such as Bax and Bak lack the BH4 domain. These members require an activation event to be able to interact with Bcl-XL or Bcl-2. Cells deficient in both Bax and Bak proteins are resistant to death stimuli that stimulate the intrinsic apoptotic pathway. Bax and Bak function together at the mitochondria as the gatekeepers of the intrinsic apoptotic

pathway [60]. Bax and Bak also reside at the ER [61] with a rather different function, which will be discussed in the Bcl-2 proteins at the ER Membrane section.

Upon a death signal, Bax inserts into the mitochondrial outer membrane (MOM) as a homooligomerized multimer. Inactive Bak also undergoes a conformational change and oligomerizes. These changes bring about the permeabilization of the MOM and the release of mitochondrial intermembrane space proteins into the cytosol. The precise mechanism of this membrane permeabilization is of yet unclear. Cytochrome c is one significant protein to the apoptotic machinery released from the mitochondria.

Another group of Bcl-2 family proteins consists of the single BH3 domain proapoptotic members. From now on, this type will be referred to as "BH3-only proteins". They serve as upstream sentinels that respond to specific death stimuli and survival signals [51]. The activity of BH3-only proteins is transcriptionally and/or posttranscriptionally controlled upon death stimuli.

Bid, for example, is posttranslationally controlled by cleavage. It is a substrate of active caspase-8 that is activated by the extrinsic pathway. Cleaved and activated Bid is called tBid. Active tBid targets the mitochondria to trigger the oligomerization of Bax or Bak [60], [62] to promote the release of cytochrome c, thus connecting the extracellular death pathway to the intracellular apoptosis machinery.

Noxa and Puma are controlled transcriptionally by the transcription factor p53 in response to DNA damage [63].

Bad is regulated by phosphorylation in response to growth and survival factors [64].

BH3 proteins have a proapoptotic effect on Bax and/or Bak, thus stimulating cytochrome c release from the mitochondria in direct or indirect ways. It is known that in the absence of Bax and/or Bak, BH3-only proteins are incapable of evoking this release. There are possible ways of how BH3-only proteins activate the prosurvival Bax and Bak.

One model proposed for the activities of BH3-only proteins functionally categorizes them into two subgroups, activators or sensitizers [65]. In this scenario, activator BH3-onlys (Bid, Bim,

PUMA) are thought to activate Bax and Bak directly. Sensitizer BH3-only proteins (Bad, Noxa) free the activators by competing to bind to the prosurvival Bcl-2 proteins.

The result of Bax and/or Bak activation through homo oligomerization is the release of cytochrome c from the mitochondria interme mbrane space. Cytochrome c helps to form a complex called the apoptosome. The main components of the apoptosome complex are cytochrome c, caspase-9 and Apaf-1 **(Figure 5).** The apoptosome, once formed initiates a caspase cascade, where the effector caspases (caspase-3) are activated in an exponential fashion resulting in cell's death. This scene roughly describes the intrinsic apoptotic pathway.

Mitochondrial membrane potential and subsequent mitochondrial membrane permeabilization that release mitochondrial proteins perpetuates the apoptotic response. Loss in the mitochondrial membrane potential is thought to mediate intrinsic and extrinsic apoptosis pathways in response to anticancer drugs, irradiation, growth factor deprivation, etc [66]. Overexpresion of Bcl-2 or Bcl-Xl inhibits the loss of mitochondrial membrane potential and membrane permeabilization inhibiting apoptotic cell death [67].

1.4.1.2 Other Molecules in the Apoptosis Pathway

IAPs (Inhibitors of apoptosis): IAPs inhibit caspase -3, -7 and -9 [68], [69]. Binding of IAPs to caspases block the access of the substrate to caspases. Smac /DIABLO and HtrA2/Omi are released from the mitochondria intermembrane space as well as cytochrome c. They bind IAPs to inhibit their prosurvival function [70]. The IAP binding domain of caspase -9 is released upon its cleavage by caspase-3. The cleaved part binds to XIAP to keep it inactive [71]. The significance of IAPs for being a checkpoint for apoptosis processing varies among different organisms.

1.4.1.3 Extracellular Apoptosis Pathway and MAP Kinases

The cell receives death and survival signals from the extracellular environment. TNF receptor gene family consisting of Fas, TNF receptor I, DR3 play s a significant role in relaying death signals from outside of the cell.

The death domain (DD) is the conserved intracellular domain of the death receptor family members [72]. Upon mitogen (TNFα) binding to the TNF receptor, the receptor trimerizes, the three death domains come together to recruit TRADD (TNF receptor I -associated death domain protein), which mediates the recruitment of the adaptor molecule, TRAF2 (TNF receptor-associated factor 2) [73]. TRAF2 recruitment activates the MAP kinases JNK, p38, and also the transcription factor NF-kB (nuclear factor for k chain gene in B cells) through the recruitment and activation of MAPKKK ASK1. A kinase-inactive mutant of ASK1 inhibits the TNFα and TRAF2-induced JNK activation. ASK1 associates wit h endogenous TRAF2 in a TNFα-dependent manner [74], [75].

TRADD also recruits FADD (Fas-associated death domain-containing protein) [76] and RIP (receptor interacting protein). FADD recruits caspase -8, initiating the apoptotic cascade. RIP recruitment activates NF-kB. FADD can also recruit RAIDD (RIP-associated ICH-1/CED-3-homologous protein with a death domain), which then recruits caspase -2.

Fas/CD95 is another member of the death receptor family. Fas/CD95 ligand activation of Fas/CD95 results in the recruitment of FADD, and the activation of caspase-8 follows. ASK1 also associates with the active Fas trimer through the adapter protein Daxx [77].

The extrinsic pathway uses the mitochondria to amplify the death signaling through the cleavage of Bid by active caspase-8 [78].

1.4.1.4 Cell engulfment, How is the Apoptotic Cell Removed?

The noninflammatory nature of apoptosis is due to the engulfment of the organism's apoptotic cell bodies by phagocytic cells. Phagocytes recognize the surfac e or the dying cell exposing an "eat me" signal [51], which is phosphatidylserine (PS) residues [79]. Phosphatidylserine residues that reside in the cytosolic side of the cell membrane flip to the extracellular side during apoptosis.

1.4.2 Apoptosis and ER Stress

The relationship of ER stress and apoptosis can be thought as the quality of the synthesized proteins serving as a checkpoint for cell death. There are many critical control steps in the intrinsic apoptosis pathway that are localized to the surface of cellular organelles [51].

The cell eventually triggers cell death mechanisms when the UPR is unable to compensate for the damage the stress exercises on the cell. The mechanisms by which ER stressed cell switches from the survival response to promote demise (typically apoptosis) are not free of doubt. There are multiple potential participants of this switch from survival to death. The dominating factors are also thought to differ by cell type.

Several reports have shown the role of mitochondria in the activated apoptotic pathway due to ER stress. In the study of Hacki et al., 2000, it has been revealed that there exists a crosstalk between the ER and mitochondria, where ER stress triggers cytochrome c release and subsequent apoptosome formation leading to caspase-dependent apoptosis [80].

It remains a question how the signaling from the ER is carried to the mitochondria. Is it that BH3-only proteins are activated at the face of prolonged UPR, are the ER stress sensor proteins IRE1, PERK, ATF6 necessary for this crosstalk, do the regulation of MAPK play a role, is calcium release from the ER a signal carrier?

Several mechanisms have been proposed for linking ER stress to cell death.

These mechanisms include the direct activation of proteases, kinases, transcription factors and Bcl-2 family proteins [81]. The pathways and their intricate relations will be discussed below.

The bZIP transcription factors activated by the UPR are mainly responsible for the execution of the survival response. They activate the 'adaptation genes' for survival [81]. IRE1, on the other hand, in addition to activating the adaptive response, also activates the genes that alarm the cell in the case of possible nonsuccess of the survival response. In this sense, it is not surprising that IRE1 shares many common molecular functions with the members of the TNF receptor family. TNF receptor family is activated in the face of viral infections that are associated with massive glycoprotein production. One commonality of the TNF receptor and IRE1 is their binding to TRAF2. TRAF2 activates the protein kinases implicated in immunity and inflammation. ASK1 is one substrate of TRAF2, which activates JNK and other kinases linked to NF-kB activation [82]. Therefore, IRE1 takes part in all the response mechanisms

during stress, XBP-1 for survival, NF-kB through TRAF2 for alarm, and JNK and p38 through ASK1 for death. This is not surprising in the light o f the fact that IRE1 is the sole UPR arm in lower eukaryotes.
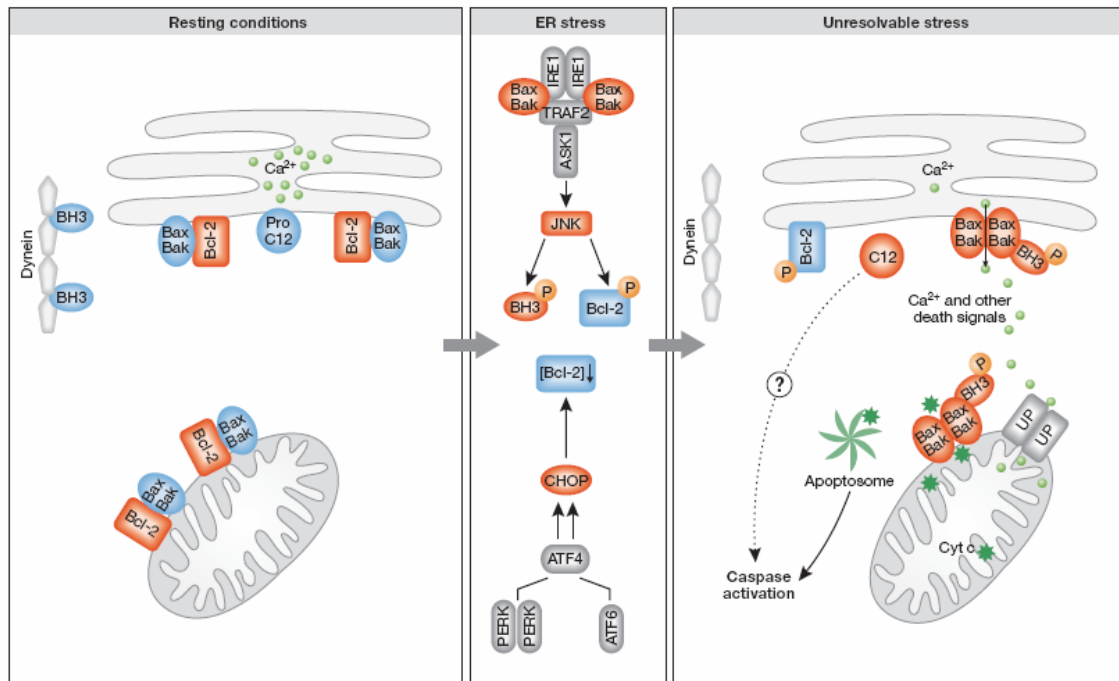
Figure 1.5 The involvement of Bcl-2 proteins in ER stress conditions.

ER stress leads to the activation of JNK through the IRE1 -TRAF2-ASK1 pathway. CHOP is induced by all three arms of the UPR. JNK inhibits the prosurvival Bcl -2 by phosphorylating it. CHOP blocks the expression of Bcl-2. JNK also phosphorylates Bim, a BH3 -only protein found bound to the cytoskeletal dynein in stress -free condition. Calcium release from the ER may be a factor relaying the death signal from the ER to the mitocho ndrial intrinsic apoptosis pathway. Blue labels represent the inactive proteins. Red labels represent active proteins. Round molecules are prodeath and rectangular molecules are prosurvival members. In rodents, caspase-12 have been shown to associate with IRE1 for activation [83]. Homolog of caspase-12 in mammals is nonfunctional [84].

1.4.2.1 ASK1

Intracellular signaling via phosphorylation (by kinases) and dephosphorylation (by phosphatases) is a common regulatory control of cellular events. Protein kinases take part in signaling that regulate life and death decisions (reference). Mitogen-activated protein (MAP) kinase cascade is evolutionarily well-conserved in eukaryotic cells and they function as mediators of cellular stress [85].They enable cells to respond to extracellular stimuli and cell stress [86]. The typical cascade is comprised of MAP kinase kinase kinase (MAPKKK) , MAP kinase kinase (MAPKK) and MAP kinase (MAPK). Regulation is carried out at each

level. The MAPK family is large. The three members of the MAPK family are JNK (c-Jun N-terminal kinase), p38 and ERK (extracellular signal-regulated kinase).

ASK1 (apoptosis signal-regulating kinase 1) is a MAPKKK that activates both the MEKK4/MEKK7-JNK and MKK3/MKK6-p38 pathways and induces apoptosis in various cell types [75], [87], [88]. Constitutively active ASK1 mutant induces cytochrome c release from the mitochondria and it fails to induce caspase -3 activation in *caspase9*-/- cells [89]. Apart from ER stress, JNK and p38 are known to be activa ted by stresses such as osmotic shock, UV radiation, heat shock, oxidative stress, protein synthesis inhibitors and Fas stimulation [90], [91].

ASK1 is reported to be required for TRAF2-dependent JNK activation in the extracellular apoptosis pathway [75]. Later on Nishitoh et al. in 2002 reported that during ER stress induced by expanded polyglutamine repeats, ASK1 associates with TRAF2 for its activation and that *ask1*-/- primary neurons are resistant to ER stress-induced JNK activation and apoptosis. However, it is possible that ASK1 may be mediating the survival -death response through not only JNK but other paths [92].

1.4.2.2 A Downstream Effector of ASK1, c-JUN terminal kinase (JNK)

JNK family of MAP kinases arise from three genes, Jnk1, Jnk2, and Jnk3. Alternative splicing at the mRNA level can give rise to 10 different JNK isoforms differing at their C -termini and substrate binding and tissue distribution [93]. IRE1-TRAF2-ASK1 pathway phosphorylates and activates JNK during the UPR. Active JNK leads to the phosphorylation and activation of most notably the transcription factor c-JUN (at residues Ser63 and Ser73) and ATF2 [94], [95].

JNKs have a role in apoptosis, but they have also been reported to promote cell survival and proliferation. The contradictory observations for the effect of JNK activation ha ve been attributed to its ability to activate a large number of different substrates based on specific stimulus, cell type, and the temporal availability of substrates [96]. It is also argued that JNK1 and 2 isoforms selectively target specific transcription factors. JNK1-/- and JNK2-/- mice show very different patterns of gene expression [97]. jnk1 or jnk2 deficient mice survive

normally, whereas the absence of both JNK1 and JNK2 causes the early embryonic death of mice associated with neuronal apoptosis defects and exencephaly [98], [99].

jnk-null MEFs do not exhibit defective Fas/CD95-induced apoptosis; however, they do exhibit defects in stress-induced apoptosis, such as UV radiation, translational inhibit or anisomycin and to DNA alkylating agent methylmethanesulfonate. These cells are defective in mitochondrial depolarization and the release of cytochrome c, required for the formation of the apoptosome and subsequent caspase activation [100].

Constitutive JNK basal activity has been reported to be elevated in some cancers. The constitutive biologic function of JNK is thought to promote cell survival and growth [101]. Wang et al. examined breast cancer and normal human breast tissues, and observed that the expression of JNK1, but not JNK2 was markedly increased in cancerous tissues [102].
In some tumor cells, inhibition of JNK2 expression by antisense oligos suppressed growth and induced apoptosis in a p53-dependent manner [103]. However, JNK function may also differ by cancer type. JNK1 may act as a tumor suppressor in skin cancer cells, whereas JNK2 may act as a suppressor in lymphomas [96].

Xia et al investigated the gastrointestinal cancer cell lines, SW1116, HT29, COLO205, BCG -823, MKN-45 and AGS for JNK activity [104]. In general, for these cells JNK2 expression was found to be stronger than JNK1 both at mRNA and protein levels. In all of these cell lines, cell viability decreased in a time-dependent manner with the administration of the JNK inhibitor SP600125. Apoptosis increased in COLO205, AGS, BCG -823 and MKN-45. Apoptosis was not the mechanism of death in SW116 and HT29 cell lines.
However, Du et al argue that the JNK inhibitor SP600125 has a cytostatic rather than a cytotoxic effect meaning that cells that are inhibited in growth would eventually succumb to apoptosis [105].

JNK expression increases upon stress, cytokines and many anti -cancer drugs [106]. Ventura et al. argue that the time course of JNK activation and the physiological context determine the effect of JNK activation in cells, reporting that early transient JNK activation corresponds to survival, whereas a late more sustained JNK activation mediates the apoptotic signaling [107].

There are several reports connecting JNK activity to the activation of the intrinsic apoptotic pathway. Co-expression of ASK1 and JNK is known to phosphorylate and inactivate the prosurvival Bcl-2 protein [108]. Moreover, Bim, a proapoptotic BH3-only member of the Bcl-2 family, is induced by the JNK- c-JUN pathway [109]. Phosphorylation and activation of Bim by JNK is important for sympathetic neuronal cell death [110]. However, JNK is also known to phosphorylate the proapoptotic BAD to inhibit apoptosis [111].

Phosphorylation and inactivation of the prosurvival Bcl-2 by JNK, upregulation of proapoptotic Bim expression through the c-JUN pathway, reduction of UV radiation-induced apoptosis in *jnk1/2*-/- cells all indicate a prodeath role for active JNK. A proapoptotic role for JNK has been described in apoptosis induced by vinblastine, doxorubicin and etoposide [112, 113].

An example to cell type specificity of JNK activity is the study of Kuntzen et al on CD95/Fas-induced apoptosis. They have also shown that JNK inhibition increases CD95/Fas-induced apoptosis in liver cells, Chang hepatoblastoma cells, HT29 colon carcinoma cells, and T-lymphoblastoid cell line Jurkat. However, JNK inhibition did not increase death in CD95-induced Huh7 or Hep3B liver carcinoma cells [114].

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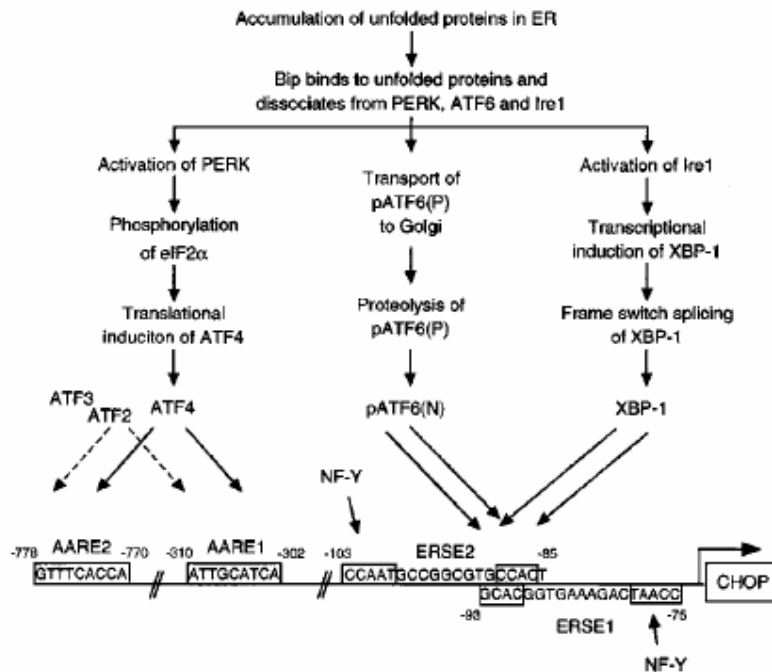
Figure 1.6 [27]. CHOP protein is induced by all three arms of the UPR.

During ER stress, the transcriptional induction of CHOP is regulated by at least four cis-acting elements, AARE1, AARE2, ERSE1 and ERSE2.

1.4.2.3 CHOP/GADD153

CHOP/GADD153 is a 29 kDa protein with 169 amino acid residues in humans and 168 in rodents. It is a member of the CCAAT/enhancer binding protein (C/EBPs) family. C/EBPs are a group of transcription factors that regulate cell differentiation and proliferation. CHOP has two known functional domains. One is the N-terminal transcriptional activation domain and the other is the C-terminal basic leucine zipper (bZIP) domain [115].

Non-stressed cells don't express CHOP or express at low levels [116]. CHOP mRNA contains a uORF sequence in its 5' untranslated region (5'UTR), which represses its translation during normal cell condition [117].

CHOP is highly induced during ER stress [118], [35]. The *chop* gene promoter contains at least two ERSE and two AARE motifs. The ERSE motifs show homology with the other ER stress activated genes, such as BiP, GRP94, PDI, and calreticulin (CRT) [119].

CHOP gene is induced by all three arms of the UP R, ATF4 through PERK [35], ATF6 and IRE1 through XBP-1. ATF6 overexpression can induce the *chop* gene; however, CHOP induction is not observed in *perk*-/- cells. Therefore, it can be said that the PERK/eIF2α signaling and ATF4 is dominant in the induction of CHOP during ER stress [35]. However, for maximal induction of CHOP, ATF6, IRE1/XBP1 and PERK are all necessary.

Overexpression of CHOP leads cells to growth arrest and apoptosis [120], [121]. CHOP has been reported as a protein involved in ER stress-induced apoptosis. Overexpression of BiP reduces CHOP induction (because it reduces PERK, ATF6, IRE1 activation) and reduces ER stress-induced apoptosis [122]. *chop*-/- mice are resistant to ER stress-induced apoptosis [27], [123].

CHOP is known to regulate the expression of Bcl-2 family genes [116]. Overexpression of CHOP leads to a decrease in Bcl-2 proteins and CHOP-induced apoptosis is blocked by overexpressing Bcl-2 prosurvival family members [124] . Overexpression of CHOP also causes the translocation of Bax from the cytosol to the mitochondria [124]. However, it is not known that the effect of CHOP on Bcl-2 proteins is direct or indirect.

GADD34 promotes the restart of the global translation [125]. CHOP appears to promote protein synthesis partly through the induct ion of GADD34 [126]. Therefore deleting the *chop* gene may be promototing cell survival through continued translational attenuation. GADD34 null animals have been shown to be protected from ER -stress related death similar to CHOP null animals [127].

1.4.2.5 Bcl-2 Protein Family at the ER Membrane

Apart from their roles as initiators/protectors of the intrinsic apoptotic pathway, Bcl -2 family proteins have recently been found to function actively in non -canonical roles at the ER membrane. Bcl-2 proteins are localized, at least partially in the ER membrane and periphery as well as the mitochondria [128].

1.4.2.5.1 Bax and Bak

bax-/- and *bak*-/- double knockout mice are resistant to ER stress-induced death [60]. This may show that the apoptotic response upon ER stress includes Bax and Bak as executioners. Bax and Bak have a role at the ER membrane for the homeostasis of ER Ca+2 [61]. In addition to being resistant to ER stress-induced death, *bak*-/-*bax*-/- double knockout mice are also defective in calcium homeostasis at the ER.

XBP-1 expression (related to IRE1) in *bax*-/-*bak*-/- cells was impaired consistent with tunicamycin injection. IRE1->TRAF2->ASK1->JNK pathway was also impaired like in the phenotype observed in *ire1*-/- cells. This phenomenon is likely not related to the imbalance of prosurvival and proapoptotic proteins since the reverse of the case was not observed in *bcl-2*-/- cells. In accordance with the observations with the effect of the absence of BAX and BAK on the IRE1 pathway activation, an alternative role for these proteins is to modulate the amplitude of IRE1 activation by controlling its autophosphorylation and oligomeriation [129].

In this case, BAX and BAK contribute to the survival adaptations of the cell during early signaling. Hetz and Glimcher speculate that the disruption of the IRE1 and Bax/Bak interaction might be a switch for the UPR survival response and BH3-only proteins might have a role in disrupting the interaction.

1.4.2.5.2 BH3-only Proteins

BH3-only PUMA and NOXA are strongly induced at transcriptional level in ER stressed cells. In SY-S5N neuronal cells, a finding by microarray analysis reveals that PUMA is the only BH3-only protein expressed during ER stress [130]. In a global RNA-interference screen for ER-stress-mediated apoptosis related genes, PUMA and NOXA have been implicated to be upregulated [131]. Accordingly, *puma*-/- and *noxa*-/- cells are partially resistant to ER stress-induced apoptosis [132].

BH3-only BIM is found in the dynein motor complex of the microtubule cytoskeleton in nonstressed cell conditions. Following ER stress, BIM translocates to the ER [133]. How BIM functions at the ER is unknown. BIM mRNA has found to be upregulated by CHOP.

bim-/- mice are resistant to ER stress-induced apoptosis in vivo, in a way similar to *chop*-/- mice [126].

The upregulation of BH3-only proteins may function to direct the signals from the damaged ER to the intrinsic (mitochondrial) apoptosis pathway [56].

1.4.2.5.3 Bcl-2

The prosurvival effect of Bcl-2 may be different depending on its localization. One group reported that Bcl-2 directed to the mitochondria is more protective against death than Bcl-2 localized at the ER when the cell is disturbed with serum withdrawal and etoposide, which are known to trigger slow mitochondrial disruption and cytochrome c release [134].

Activation (dephosphorylation) of Bcl-2 by the serine/threonine phosphatase PP2A [135] and inactivation (phosphorylation) of Bcl-2 by JNK regulates its functioning at the ER membrane.

1.4.2.6 Role of Calcium

One of the main functions of the Bcl-2 family proteins at the ER is controlling the calcium homeostasis. Calcium release/uptake of the ER happens through the ryanodine receptor and the inositol triphosphate receptor (IP3R). Bcl-2 and Bcl-xl form a protein complex with IP3R modulating its on-off state [136].

Calcium released from the ER can be taken up by the mitochondria through a low-affinity uniporter, acting like a buffer for calcium signals [137]. In this sense, in addition to the upregulation of BH3-only proteins, modifications of Bcl-2 by JNK, induction of the apoptosis moderator CHOP during ER stress; calcium may act as a mediator of the ER-mitochondria crosstalk to relay the apoptotic signals. Calcium uptake by mitochondria induces the mitochondrial membrane permeabilization and opening of the permeability-transition pore (PTP) and cytochrome c release.

Calcium activates cytoplasmic proteases such as calpains that can cleave caspases [138]. Calcium/calmodulin-dependent phosphatase can dephosphorylate and activate the proapoptotic BH3-only protein BAD.

The balance between the prosurvival and proapoptotic Bcl-2 family proteins and post-translational modifications as such at the ER membrane may regulate the calcium release from the ER. Accordingly, calcium release from the ER is triggered by the overexpression of BH3-only proteins [139].

Calcium released from the ER can regulate functions such as cell cycle entry, proliferation, differentiation and cell death. 2003 paper by Scorrano et al. argue for regulation of calcium by bax and bak as the candidate for control point of apoptosis [61].

1.4.2.7 ERK MAPK

ERK1/2 MAPK phosphorylates and activates various transcription factors and other protein kinases. ERK1/2 plays a role in cell survival, differentiation and cell cycle-regulation. ERK is a MAP kinase not activated by ASK1 unlike JNK and p38.

The study by Arai et al., 2004 connects ER stress in SH-SY5Y neuroblastoma cells with MAP kinase signaling through ERK. SH-SY5Y neuroblastoma cells treated with thapsigargin, the major MAPK that was phosphorylated in response was found to be ERK-1 and -2, with its peak activation occurring around 30 min to 1hr. There was no significant increase in the phosphorylation of JNK or p38 in these neuroblastoma cells [86].

Inhibition of ERK phosphorylation by the MEK inhibitors U0126 and PD98059 made cells resistant to thapsigargin induced-cell death in a dose-dependent manner, measured by the release of LDH (lactate dehydrogenase) into the medium. However, pre-treatment with U0126 did not decrease the levels of cleaved caspase-3 or the caspase substrate PARP. Pre-treatment with the pan-caspase inhibitor Z-VAD-FMK decreased the levels of cleaved caspase and PARP and LDH release into the medium. However, death was not fully inhibited [86]. Inhibition had a protective role in these cells, caspase-3 activation was not diminished. What can be concluded is that ERK activation contributes to cell death either in a caspase-independent fashion or at least downstream of caspases in this cell line.

Lawrence et al have examined ERK1/2 effect on CHOP expression in response to the ER stressors thapsigargin and tunicamycin in pancreatic Beta -cells. They have observed that ERK1/2 inhibitor U0126 inhibited CHOP expression in duced by thapsigargin. The activity of CHOP 5' promoter was markedly inhibited by ERK1/2 inhibition in response to thapsigargin treatment, and partially inhibited in response to tunicamycin treatment [140].

Classically, ERK activation belongs to the group that promotes cell survival [141]. An example is the prevention of apoptosis by ERK activation in cerebellar neurons and PC12 cells upon growth factor withdrawal [142]. However, ERK activation was deleterious after excitotoxic, traumatic and ischemic insults in neurons and the brain in vivo in some studies [143].

The contribution of ERK activation to cell death seems to be cell type and stimulus-specific.

1.4.2.8 PI3K/Akt Pathway in General and in ER Stress

PI3K is a pathway that promotes cell survival by withstanding apoptotic insults [144]. An important functioning for the PI3K pathway is inhibition of apoptosis. Akt is a serine/threonine protein kinase that is mainly regulated by PI3K.

Akt is identified as being a protective element that responds to apoptotic stimulus in favor of cell's survival. Akt protects cells from different kinds of apoptotic insults, such as growth factor withdrawal, Fas-induced apoptosis, oxidative stress, UV irradiation, DNA damage, etc [145], [146].

Akt is known to inactivate proapoptotic elements in the cell, as discussed below.

A pro-survival effect of Akt is the inhibition of Bax conformational change that promotes the release of cytochrome c [147].

Akt is known to phosphorylate the proapoptotic BH3 only protein BAD on Ser136, neutralizing its proapoptotic effect [148]. Active, nonphosphorylated Bad functions as a proapoptotic protein. It binds and neutralizes the prosurvival Bcl-2.

However, Akt can protect cells even in cells lacking BAD expression.

Akt phosphorylates and inhibits ASK1 [149], inactivates Forkhead transcription factors [145], and caspase-9.

In the study by Hu et al., Akt is activated in response to ER stress in MCF-7 breast cancer cells, H1299 human lung cancer cells and PC-3 human prostate cancer cells [150]. A speculation is that PI3K is activated as a response to increased intracellular calcium induced by ER stress. Inhibition of Akt activity by the PI3K inhibitor LY294002 sensitizes MCF-7 and H1299 cells to tunicamycin and thapsigargin-induced cell death.

MAPK ERK was also induced in response to tunicamycin and thapsigargin treatment in MCF-7 and H1299 cells and the inhibition of ERK by U0126 increased cell death induced by ER stress.

Akt inhibition caused a decrease in the levels of phopshorylated ERK, the reverse of the case was not observed. It is possible that the activation of ERK is dependent on active Akt.

In the same study, two proteins cIAP-2 and XIAP that belong to the IAP family of caspase suppressors are found to be upregulated during ER stress in relation to the PI3K/Akt pathway. The inhibition of PI3K by the inhibitor LY294002 or by dominant-negative Akt decreased the levels of these IAPs, making cells more prone to cell death. This is sensible since IAP family proteins have survival functions, presumably by through inhibiting caspase activity.

Treatment of cells with caspase inhibitors decreased cell death to an extent; therefore, the levels of significant proteins in caspase-dependent cell death are assayed. The study shows that there is no translational difference of the Bcl-2 protein family members such as Bax, Bak, Bid, Bim and Bcl-2. However, the ablation of IAPs increase sensitivity to cell death and this scenario is does not hold true when caspases are inhibited.

The hypothesis tested in this study is that in parallel to the activation of UPR, stress activates cell survival mechanisms that counteract apopotic signals to facilitate the cytoprotective function of the UPR and that survival elements directly counteract ER stress-induced apoptotic signaling. They show that ERK and Akt are activated in the face of ER stress and

their inactivation sensitizes cells to ER stress and that the upregulation of caspase suppressor IAPs by ER stress are in part mediated by Akt.

1.5 Cell Cycle Regulation

The activity of regulatory cyclins and their catalytic partners, the cyclin -dependent kinases (CDKs) are required for cell cycle progression. CDKs are activated by cyclins. Cyclin - dependent kinases (CDKs) are necessary for orderly transition between these stages.

Progression through the G1 phase initially depends on holoenzymes composed of D type cyclins (D1, D2 and/or D3) in association with either CDK4 or CDK6. CDK4 is activated during mid-G1. CDK4 activity depends on proteins of the cyclin D family. For cells to undergo the G1-> S transition, the retinoblastoma tumor suppressor protein (pRB) must be inactivated. The inactivation of pRB is achieved by CDK4-mediated phosphorylation.

The ability of pRB to suppress G1 to S transition is related to pRB sequestration of the sequence-specific transcription factor E2F. When pRB is phosphorylated and inactivated by CDK4 and CDK6, E2F is released. E2F and DP1 (DRTF1 polypeptide -1) promotes gene expression required for the G1 to S transition. These genes include cyclin E (induced late in G1). Cyclin E binds and activates CDK2 as cells approach the G1/S transition [151]. The sequential activation of cyclin-dependent kinases promotes the continuity of the cell cycle.

Mitogen deprivation or anti-proliferative cytokines stop the cell-cycle through the degradation of unstable cyclin subunits by specific post-translational modifications of the CDK subunits, or via association of active CDKs with polypeptide CDK inhibitors (CKIs).

CKIs are upregulated in response to anti-proliferative signals. P16 INK4A is an inhibitor of cyclin D - CDK4 complexes. It prevents the inactivation of pRb.

Cip/Kip CKIs (including p21Cip1, p27Kip1, and p57Kip2) are potent inhibitors of cyclin E - CDK2 and cyclin A - CDK2 complexes. They positively regulate cyclin D – CDK complex when bound to it [152, 153]. p27KİP1 inhibits the cyclin E – CDK2 complex. p27KİP1 is degraded by the ubiquitin/proteasome pathway. If not, cells cannot transit out of late G1. The

ubiquitin aided degradation of p27KİP1 depends on its phosphorylation by the cyclin E-CDK2 complex.

p21CİP1 is induced by p53-dependent (due to DNA damage) [154] and p53-independent (due to stress response or differentiation) [155] pathways. It inhibits both CDK4/6 and CDK2 [156] complexes by binding them causing cell cycle arrest at the G1 phase.

p21 can be regulated posttranscriptionally [157]. p53-independent regulation of p21 depends on MAPKs. p21 has a short-half life of about 20 to 60 minutes in most cells. p21 is also degraded by the ubiquitin/proteasome pathway [158]. p21 was shown to be upregulated by p53. Liu et al have shown that regulation of p21 in response to mitogenic stimulation is not dependent on p53 [159].

Another study showed that it has an inhibitory effect on cyclin B − CDK2 complex and plays a role in G2-M transiton [160].

Mitogen withdrawal inhibits cyclin D1 transcription, which accelerates the turnover of the short-lived protein causing the disassembly of cyclin D − CDK complexes. The disassembly releases the Cip/Kip proteins associated with the complexes. Free CKIs now can inhibit cyclin E - CDK2 and cyclin A - CDK2. This kind of inhibition prevents S phase entry and results in G1 phase arrest [161]. Enforced ectopic expression of cyclin D proteins in this context resequesters the Cip/Kip proteins and enables S phase entry.

1.5.1 Cell Cycle Arrest During ER Stress

Even though the CKIs break the cyclin A - CDK2 and cyclin E - CDK2 complexes, they positively regulate the cyclin D - CDK(4/6) complexes [153]. Cyclin D is essential during the early timepoint of G1 to S transition. The UPR halts protein translation via the phosphorylation of the elongation factor eIF2α by PERK. PERK links the stress in the ER to the cell-cycle regulation program [162]. The decrease in the rate of overall protein synthesis effects the translation of cyclin D1 mRNA and causes the disassembly of the cyclin D1 − CKD4/6 complex.

The free CİP/KİP proteins that bind the cyclin D1 - CDK2 complex are now free to inactivate the cyclin E – CDK2 and cyclin A – CDK2 complexes [162]. This process contributes to the G1 arrest observed during the UPR.

Tunicamycin treatment of NIH 3T3 fibroblasts causes a block of cyclin D1 translation (due to an arrest in global translation) and decline in cyclin D- and E-dependent kinase activities. G1 phase arrest is observed upon TM treatment. The loss of the cyclin D1 in cell treated with tunicamycin isattributed to toinhibition of translation via the phosphorylation of eIF2α [38]. PERK overexpression fails to arrest cell cycle that overexpress the proteasome resistant cyclin D1 mutant or in Rb-/- MEFs that do not require cyclin D1 for proliferation [162].

For a complete cell cycle arrest at G1 phase, it is required that cyclin A - and E-CDK2 complexes are inactive as well. Cyclin E and cyclin A protein amounts are not as much reduced as cyclin D1 upon overexpression of PERK or TM treatment. However, p21Cip1 protein that is freed upon cyclin D loss (cyclin D1 - CDK4 complex loss) binds to cyclin A and E-CDK2 complexes, inhibiting their activity. The level of p21 Cip1 protein that coprecipitates with CDK2 increses with overexpression of wild-type PERK or tunicamycin treatment [162].

Overexpression of cyclin D1 causes maintenance of cyclin D and E dependent kinase activities. By overexpressing cyclin D1, ER-stressed cells can be kept in cycle although a fully-activated UPR is taking place.
Overexpression of wt cyclin D1 or mutated cyclin D1 (alanine to threonine substitution at codon 286. phosphorylation of Thr-286 by glycogen synthase kinase-3Beta (GSK-3Beta) triggers the ubiquitination and proteasomal degradation of cyclin D1, the mutant cyclin D1 is highly stable) in serum-starved cells that is associated with mitogen loss does not render cells resistant to cell cycle arrest. However, the overexpression of both wt and mutant cyclin D1 in cells treated with tunicamycin leads the cells pass the G1 phase. There is a general role for cyclin D1 loss in the UPR for cell cycle arrest.

Control of cell cycle is performed at the G1 -to-S transition, G2 and M phases.

1.5.2 MAPK's in Cell Cycle

The activity of the extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) is required for cyclin D1 expression in NIH 3T3 cells. Serum starvation (mitogen loss) in these cells causes the dephosphorylation of ERK1 and ERK2 MAP kinases.

For cell cycle to progress, sequential activation of cyclin-dependent kinases (CDKs) is necessary. p21 WAF1/CIP1 protein inhibits cell cycle progression via interacting with cyclin-CDK complexes [156]. p21 has a half-life of 20-60 minutes in most cells [163].

JNK is a MAPK with a role in cell cycle. There are studies showing JNK both playing a role in cell cycle progression and cell cycle arrest. In human T-lymphocytes JNK1 activation correlates with JNK1's dissociation from the p21/JNK1 complex [164].
JNK is known to stabilize p21 by phosphorylating it at Ser130 both in vivo and in vitro [165].

Study by Fan et al. show through overexpression and inhibition studies that JNK does not affect the translational regulation of p21 that overexpression of JNK1 inhibits the ubiquitination of transfected p21. They conclude that p21 expression levels is regulated post-transcriptionally by JNK activity [166]

Does ERK stabilize p21? In A431 JNK inhibition during As2O3 treatment increases the levels of p21. Conversely, it is ERK inhibition that causes attenuation in p21 protein levels. Inhibition of ERK also decreases the number of cells in in sub-G1 phase (apoptotic), wherease JNK inhibition increases the percentage of apoptotic cells [167].

2. AIM OF THE STUDY

- Investigating the effect of ER stress in terms of cell cycle regulation and cell death in HCT116 colon carcinoma cells.
  - Making the distinction of the survival and de ath phases of HCT116 cells upon ER stress.
  - Assessing the role of MAP kinase JNK in ER stress-related cell cycle arrest and cell death in HCT116 cells in comparison with the p38 a nd ERK pathways.
  - Understanding the mechanisms by which HCT116 cells die upon ER stress.

3. MATERIALS AND METHODS

3.1 Materials – Chemicals, Antibodies, Kits, Equipment

All chemicals, antibodies, and specialty material such as membranes, protein markers used in this study are listed in Appendix A.

All kits used are listed in Appendix B.

All equipment used for general laboratory procedures is listed in Appendix C.

3.1.1 Buffers and Solutions

10X PBS: 80 g NaCl, 2.25 g KCl, 23.27 g $Na_2HPO_4.12H_2O$ and 2.05 g KH_2PO_4 in 1 L ddH_2O, pH 7.4.

1X PBS-T: 10X PBS was diluted, 0.2% Tween20 was added.

Annexin V-FITC Incubation Buffer: 10 mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$ in 500 ml ddH_2O.

PI Solution: 2 ml PBS, 12 µl TritonX100, 10 µl RNase A, 20 µl PI added to 1 ml of solution for 10 samples.

2.1.2 Buffers for SDS Polyacrylamide Gel Electrophoresis and Western Blotting

Running Buffer (10X): 250 mM Tris base, 1,92 M Glycine, 1% SDS in 500 ml, pH adjusted to 8.5.

Transfer Buffer (10X): 15.41 g Tris, 72.1 g Glycine in 500 ml.

Transfer Buffer (1X): 10X transfer buffer was diluted, 20% methanol was added prior to use.

Mild Stripping Buffer: 15 g Glycine, 1 g SDS, 10 ml Tween20 in 1 L. pH adjusted to 2.2.

3.2 Methods

3.2.1 Cell Culture

wt and *puma*-/- HCT116 colon carcinoma cells were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated FBS, 100 IU/ml penicillin and streptomycin, and 2mM glutamine. Cultures were maintained in 37°C in a humidified 5% CO_2 atmosphere. For experiments cells were seeded in 6-well culture plates (200,000 cells/well), 12-well culture plates (100,000 cells/well), 60 mm culture dishes (400,000 cells/well) or 96-well culture plates (10,000 cells/well). The cells were treated at about 60-70% confluency for death assays and protein isolation and at 40% confluency for cell-cycle assays. DMSO (= 0.1% v/v) was added to all control wells in each experiment. For cyropreservation, cells were trypsinized and resuspended in 1 ml 10% DMSO in heat-inactivated FBS (freezing medium). The cells collected in cyrovials were frozen at -80°C for 24 hours and then stored in liquid nitrogen until thawing.

3.2.2 Total Protein Isolation

Treated and control HCT116 cells were harvested, washed in ice-cold PBS and lysed on ice in a solution containing 20mM Tris-HCl (pH 7.5), 150 mM NaCl, Nonidet P-40 0.5% (v/v), 1 mM EDTA, 0.5 mM PMSF, 1 mM DTT and protease inhibitor cocktail (Roche, Mannheim, Germany) and phosphatase inhibitors (Phosphatase inhibitor cocktail 1 and 2 Sigma, Darmstadt, Germany). After lysing the cells for 30 minutes on ice, cell debris was removed by centrifugation for 10 min at 13,200g. Protein concentrations were determined with Bio-Rad Dc Protein assay.

3.2.3 Immunoblots

Proteins (30 µg) were separated on a 10 or 12% SDS-PAGE and blotted onto PVDF membranes. Membranes were blocked with 5% (w/v) dried milk in 1X PBS-Tween20 (0.2%) and incubated with primary antibody in antibody buffer containing 5% (w/v) dried milk in 1X

PBS-Tween 20 (0.2%) overnight at 4°C. After primary antibody incubation, membranes w ere washed for 3 * 5 min in 1X PBS-T. Secondary antibody incubation (horseradish peroxidase (HRP)-conjugated from Cell Signaling) was done in antibody buffer containing 5% (w/v) dried milk in 1X PBS-T for either 2 hours at room temperature or overnight at 4°C. Membranes were washed 3 * 5 min before proteins were analyzed using an enhanced chemiluminescence detection system (ECL-Advanced, Amersham Pharmacia Biotech, Freiburg, Germany) and exposed to Hyperfilm-ECL (Amersham Pharmacia Biotech, Freiburg, Germany).

3.2.4 Apoptosis and Cell Death Assays

3.2.4.1 MTT Assay

Cell death was determined using an MTT kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. Briefly, HCT116 cells were seeded in 96-well plates and treated. 10 µl of MTT labeling reagent was added to each well and incubated for 4 hr. Cells were then incubated in 100 µl of the solubilization solution for 12 hr. The absorbance was measured with a microtiter plate reader (Bio-rad, CA, USA) at a wavelength of 550 nm and a reference wavelength of 650 nm. Percent viability was calculated as OD of drug-treated samples/OD of control samples * 100.

3.2.4.2 Tryphan Blue Method

Cells were seeded onto 12-well plate. After treatments, cells were trypsinized and resuspended in complete medium to an approximate concentration of 100,000-200,000 cells/ml. 0.5 ml of cell suspension and 0.1 ml of 0.4% Trypan blue dye were mixed and incubated for 5 min at room temperature. 10 µl of the solution was transferred to an haemocytometer, and counted under a light microscope. Blue cells identify the dead cells. The percentage of death was calculated by taking the ratio of blue cells to all cells counted.

3.2.4.3 Annexin-V Binding Assay

Cells were seeded on 12-well plates (100,000 cells/well) and treated with tunicamycin after 24 h. After incubation, cells were detached by trypsinization, transferred to flow-cytometer tubes and centrifuged at 300g for 5 min. The supernatant was removed and cells were washed with 1 ml of 1X PBS. Cells were centrifuged for a second time. PBS was discarded and the pellet was resuspended in staining solution of 100 ul incubation buffer and 2 ul Annexin V-FITC. Cells were incubated in the solution for 15 min in dark at room temperature. In order to slow down the reaction, 500 ml of incubation buffer was added to each tube after 15 min. Cells were then analyzed by FACS.

3.2.4.4 Propidium Iodide (PI) Cell-Cycle Analysis

Cell cycle analysis was performed by PI staining using the flow cytometer. Cells to be analyzed were seeded on 12-wll plates (100,000 cells/well). Cells were treated after 24 h with tunicamycin and various MAPK inhibitors. After incubation, cells were detached by trypsinization, and washed with 1X cold PBS. The supernatant was discarded and the pellet was gently vortexed. 5 ml of 70% pure grade ethanol was added and kept at 4C. After the collection of all samples, cells were left at room temperature for 15 min, spinned down at 300g for 5 min. Cells were then washed with 1X cold PBS, supernatant was removed and 100 ul of PI solution was added to each sample. Samples were left in the dark for 45 min for dye incubation after which 500 ul of PBS was added to slow down the reaction. Samples were analyzed by FACS.

4. RESULTS AND DISCUSSION

4.1 Results

4.1.1 - Effect of Tunicamycin Concentration on Cell Death : Tunicamycin induces ER stress and the UPR by blocking asparagine-linked glycosylation of nascent glycoproteins at the ER lumen [18]. Persistent ER stress leads to cell death, typically via apoptosis [27].

To assess the cell death induction profile, wt HCT116 cells were treated with the ER stressor tunicamycin at increasing concentrations with treatment periods of 12, 24, and 48 h. Cell death was assayed by the MTT cell proliferation assay **(Fig 1A, B, C).** MTT assay quantifies cell viability. The yellow tetrazolium salt MTT is cleaved to purple formazan crystals by metabolically active cells. The formazan crystals are solubilized and color-quantified by a spectrophotometer (https://www.roche-applied-science.com/pack-insert/1465007a.pdf).

Cells did not show a significant death response in the first 12 h of treatment. This is sensible granting that the UPR is primarily a survival response. Cells die in the case that the stress is not alleviated. Significant death occurred after 24 h of treatment. The concentration of 1 µg/ml tunicamycin was sufficient to activate the death process after 24 h of treatment. This is consistent with previous reports that use the drug tunicamycin as an ER stressor.
Increasing drug concentration by nine-fold did not significantly affect viability compared to low doses. The drug tunicamycin has been reconstituted in DMSO. DMSO vehicle control of 1 µg/ml of TM (= 0.1% v/v) did not cause a significant death response, which is important for the assessment of death associated with tunicamycin. The ratio of DMSO- to TM-associated death decreases as the drug concentration increases. The increment in tunicamycin concentration does not have a significant effect on cell death, while increased DMSO concentration does.

1 µg/ml tunicamycin was chosen for further experimentation since at this concentration, DMSO in the medium does not significantly affect the cells and that it takes two days for treated cells to die, making the survey of the different phases of the UPR related to survival and death manageable.
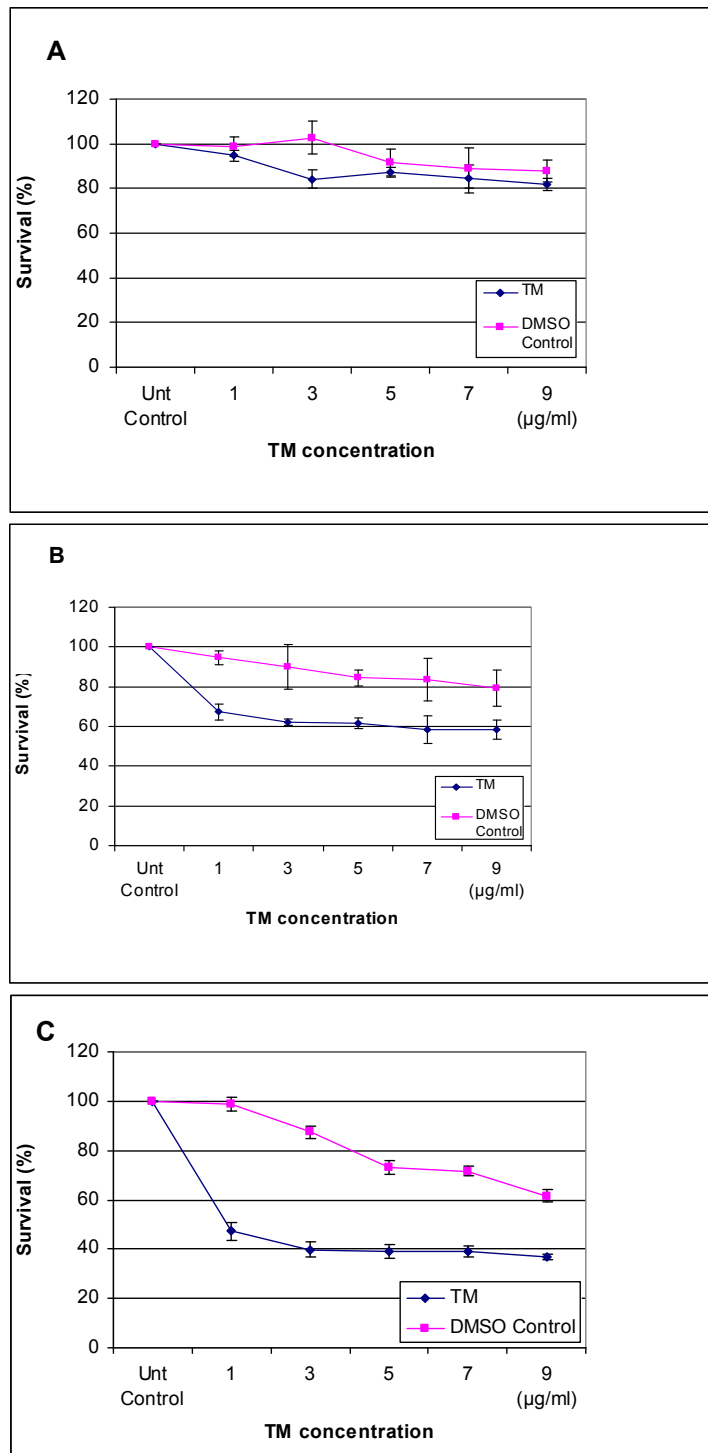
Figure 4.1 Tunicamycin dose determination.

wt HCT116 cells were treated with different doses of TM and corresponding DMSO vehicle controls for different time periods for the purpose of dose determination. Percentage survival was quantified by MTT assay. Untreated control cells were taken as 100% viable. **A,** 12 hr treatments. **B**, 24 hr treatments. **C**, 48 hr treatments.

4.1.2 - Tunicamycin Induces the Unfolded Protein Response (UPR) in HCT116 Cells : A characteristic feature of the UPR is the transcriptional increase in the levels of the transcription factor CHOP protein [123]. Microarray studies revealed that CHOP is one of the highest inducible genes during ER stress [118].

CHOP induction in HCT116 cells is observed at the first three hours of treatment with tunicamycin (1 µg/ml) **(Fig 2)**. Control cells do not express CHOP. CHOP is induced the most after 12 h of treatment. The level decreases after 24 h.
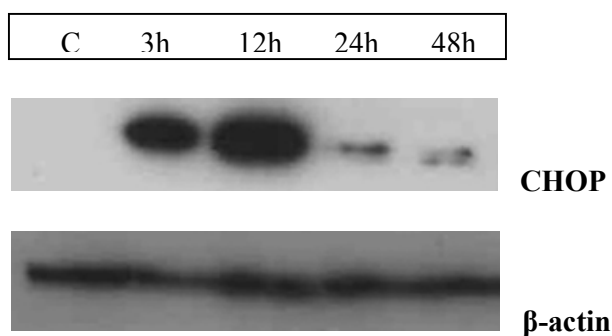
Figure 4.2. TM induces CHOP expression.

A, wt HCT116 cells were treated with TM (1 µg/ml) for various periods (0, 3, 12, 24, 48 h) and 30 µg of total protein isolates were run on 10% SDS -PAGE gel, and were subjected to Western blot analysis for CHOP protein detection and β -actin as loading control.

4.1.3 - Tunicamycin (1 µg/ml) Induces Cell Death After 24 h of Treatment, Assessed by the Trypan Blue Exclusion Method : Dead cells take the Trypan blue dye in due to loss of membrane integrity. The ratio of blue cells to all cells counted on a haemocytometer reveals the percentage of dying cells. 1 µg/ml tunicamycin -treated HCT116 cells did not lose their membrane integrity at 15 h of treatment, consistent with the results of the MTT assay. At 27 h cells, about 20% of the cells had lost their membrane integrity **(Fig 3).** Results are an average of three separate counts of approximately 100 -150 cells.
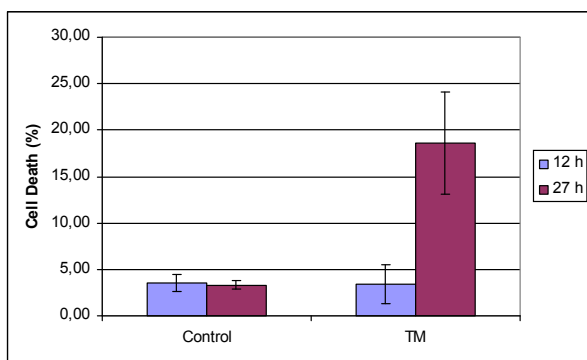
Figure 4.3. TM induces cell death assessed by the Trypan blue cell count.

wt HCT116 cells were incubated without (control) or with tunicamycin at 1 μg/ml for 15 and 27 h. TM-related cell death does not start after 15 h of treatment. Death of TM -treated cells significantly increases after 27 h of treatment.

4.1.4 - Effect of Tunicamycin on Cell Morphology : Cells were photographed under a phase-contrast filtered light microscope (photomicrography) at 100 and 400X magnification after 15 h and 27 h treatments along with control cells at 15 h **(Fig 4)**. Control cells are seen at their confluent state. Nuclei of control and tunicamycin -treated cells are visible. There is no discernible morphology change (ocular limits defined by light microscopy) in cells treated with tunicamycin after 15 h.

Reduced confluency of tunicamycin -treated cells **(Fig 4B, E)** compared to control cells **(Fig 4A, D)** after 15 h of treatment is not due to death, since these photos represent the sample cells subjected to the Trypan blue exclusion experiment under the previous title. The Tryphan blue experiment suggests that the ratio of dead cells associated with tunicamycin treatment at 15 h is no different than the untreated control sample. A probable explanation is the G1 cell -cycle arrest of cells prompted by tunicamycin. To test this hypothesis, we evaluated the ce ll cycle condition of tunicamycin -treated cells after 12 and 24 h treatments.
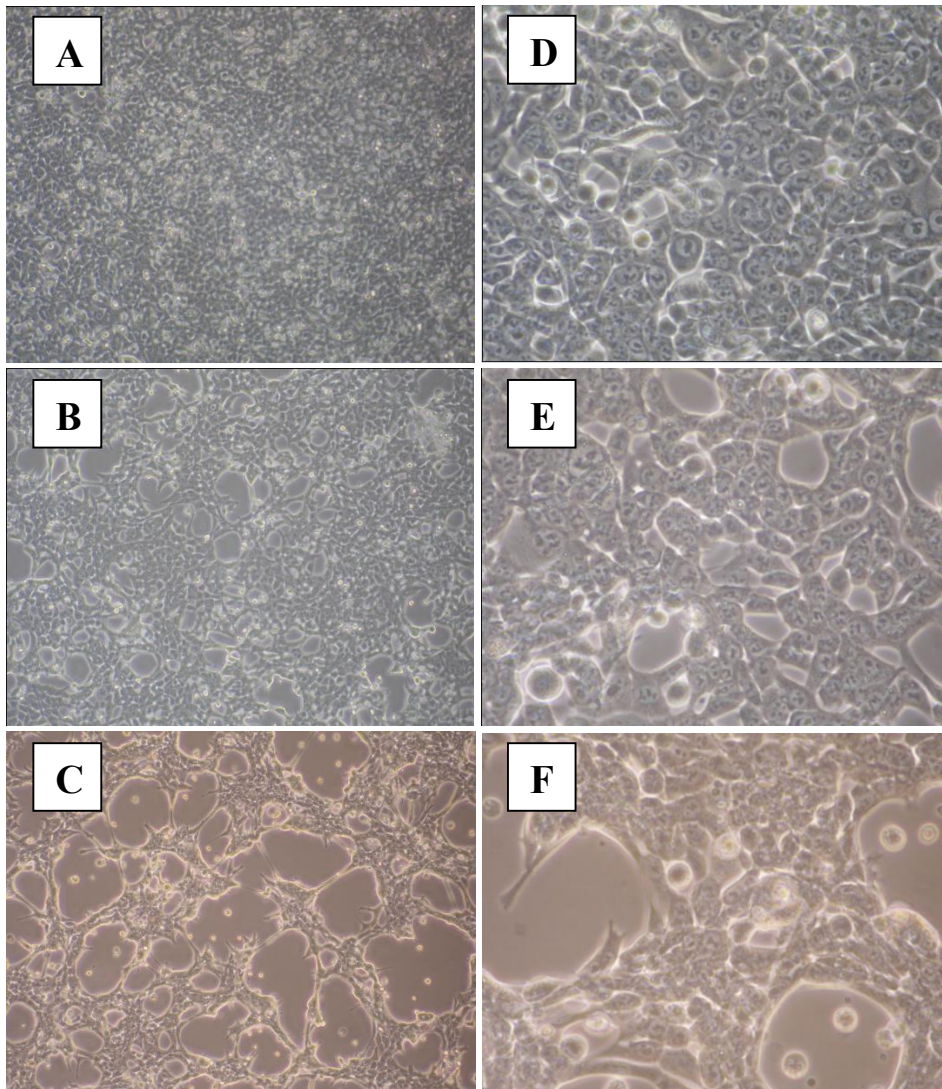
Figure 4.4. Phase-contrast photomicrography.

Photos A, B, and C were taken at 100X, and D, E, and F at 400X magnification. A and D are photos of control cells at 15 h. B and E are photos of 1 µg/ml TM-treated cells at 15 h, and C and F at 27 h. Cell death associated with TM treatment is observed in C and F.

4.1.5 - Effect of Tunicamycin on Cell Cycle: Proliferation of animal cells is controlled by the interplay between growth-promoting and growth-limiting signals. The maintenance of tissue homeostasis and control of cell number is achieved through restraints on cell proliferation and induction of programmed cell death [168]. The uncontrolled growth of cancer cells in many cases is related to the deregulation of the elements of the cell cycle.

The mammalian cell cycle is divided into two fundamental parts, the interphase and mitosis. The interphase is divided into three discrete steps. During the G1 phase, cells get ready to duplicate their DNA. DNA synthesis occurs in the S phase. During the G2 phase, cells contain 4n chromosomes and are ready for mitosis.

A-

	% G1	% S	% G2/M
Control (12 h)	46,1	16,9	37
TM (12 h)	58,2	11,4	30,4
Control (24 h)	59,8	10,5	29,7
TM (24 h)	60,4	9,9	29,7

B-

p21CİP1

β-actin
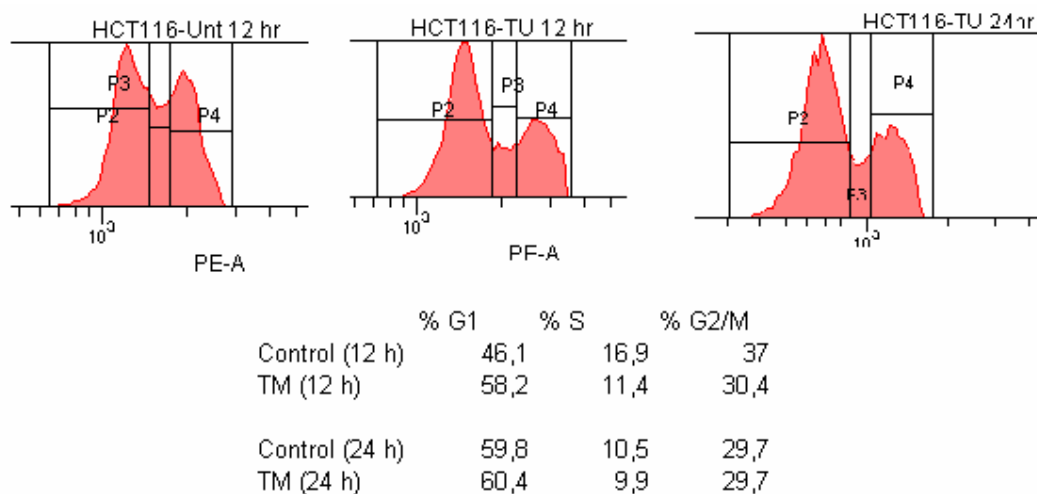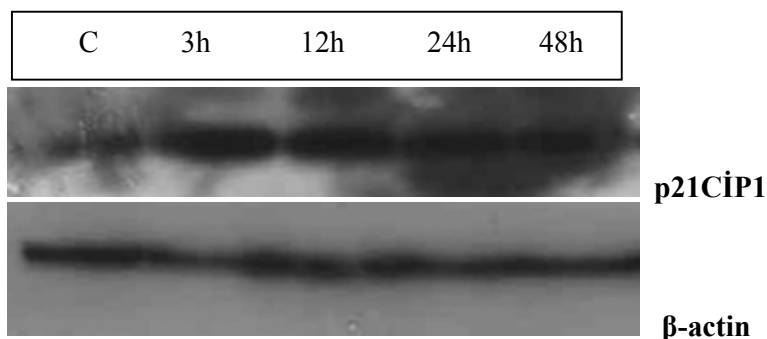
Figure 4.5A. TM induces cell cycle arrest at the G1 phase.

100,000 HCT116 cells were seeded on 12-well plates and incubated with TM for 12 or 24 h. The cells were collected and subjected to PI dye for cell cycle analysis by FACS. Figure shows the histograms for PI radiation of control and TM-treated cells. Percentage of cells at the G1 phase increase with tunicamycin treatment.

Figure 4.5B. TM increases p21CİP1 expression.

wt HCT116 cells were treated with TM (1 µg/ml) for various periods (0, 3, 12, 24, 48 h) and 30 µg of total protein isolates were run on 10% SDS-PAGE gel, and were subjected to Western blot analysis for p21CİP1 protein detection and β-actin as loading control.

Propidium iodide (PI) is a fluorescent molecule that stains DNA. The intensity of signal from PI stained DNA from a single cell analyzed by FACS is used to determine the DNA content

of the cell. The DNA content depends on the phase of the cycle the cell is in. **Figure 5A** shows the histogram of cells subjected to PI dye. The first peak identifies the cells in the G1 phase, the intermediates the S phase and the second peak the G2 phase.

Tunicamycin treatment after 12 h increases the percentage of cells in the G1 phase compared to the control sample. The ratio of G1 to G2 stays similar after 24 h.

For the orderly transition between phases of the cell cycle, the activity of cyclins and the sequential activation of their catalytic partners, the cyclin -dependent kinases (CDKs) are necessary. CDKs are inhibited by CDK inhibitor proteins (CKIs) including p21CİP1 and p27KİP1. Cell cycle arrest can be caused the degradation of cyclin subunits, or to the induction of CDKIs.

Even though the CKIs break the cyclin A - CDK2 and cyclin E - CDK2 complexes, they positively regulate the cyclin D - CDK4/6 complexes [153], which are essential during the early timepoints of the G1 to S transition. The UPR induces cell cycle arrest by blocking the translation of cyclin D1 mRNA during the gl obal translational attenuation caused by the phosphorylation of the elongation factor eIF2σ via PERK kinase activity [162]. In the case of Cyclin D1 translational attenuation, CİP/KİP proteins are free to bind the cyclin E – CDK2 and cyclin A – CDK2 complexes to inactivate them. This process contributes to the G1 arrest observed with tunicamycin treatment.

p21CİP1 is induced by p53-dependent (due to DNA damage) [154] and p53-independent (due to stress response or differentiation) [155] pathways. It inhibits CDK4/6 and CDK2 by binding them causing cell cycle arrest at the G1 phase [156].

Western blot results reveal that the expression of p21CİP1 increases with tunicamycin treatment, with the highest induction after 3 h of treatment **(Fig 5B).** Decrease in the level of p21CİP1 protein after 24 h of treatment coincides with the initiation of t he apoptotic signaling pathway **(Fig 8)**.

4.1.6- Effect of JNK Inhibition on p21CIP1 Protein Levels In Tunicamycin -treated HCT116 Cells: c-Jun NH2-terminal kinase (JNK) is a member of the mitogen -activated protein kinase family (MAPK). JNK was first iden tified as a stress-activated protein kinase. JNK has a role in the induction of apoptosis, but it also has been implicated in cell survival and proliferation. The absence of both JNK1 and JNK2 causes the early embryonic death of mice associated with neuronal apoptosis defects and exencephaly [98, 99].

JNK1 and JNK2 have differential phosphorylation and upregulation profiles. Several tumor lines possess constitutively active JNK (one or the other, or both) [169]. JNK has numerous substrates; one of many is c -JUN.

JNK is known to contribute to cell cycle progression. JNK1 and JNK2 seem to take over different tasks. JNK1 deficiency res ults in a reduction in cell number compared to wt mouse fibroblasts, whereas JNK2 deficiency results in a larger number of cells. JNK2 -/- fibroblasts spend less time in G1 compared to wt or JNK1 -/- fibroblasts [99].
ER stress-induced activation of JNK is dependent on the ER transmembrane protein IRE1. JNK is activated through the IRE1 -TRAF2-ASK1 pathway during the UPR [92].

SP600125, an anthrapyrazole, is a selective inhibito r of JNK catalytic activity [170]. SP600125 inhibits both JNK1 and JNK2 in cells. The molecule SP600125 was used at 20 μM concentration as an inhibitor of JNK phosphorylation in the experiments. According to the datasheet, this dosage efficiently inhibits JNK1 (p46) and 2 (p54) activation a nd has no effect on the MAP kinases, p38 or ERK1/2. Inhibition of JNKs via SP600125 causes the cell cycle arrest of COLO205 and AGS gastrointestinal cancer cells at G2/M phase and induces the expression of cyclin B and the CKI p27KİP1.

Our results indicate that active JNK1 (46 kDa), but not JNK2 (54 kDa) is present in non -treated HCT116 cells. Tunicamycin treatment increased the phosphorylation of JNK1 in a more pronounced fashion than JNK2. JNK1 and JNK2 are at their highest level at 12 and 24 h of tunicamycin treatment**.** The protein level of inactive, non -phosphorylated forms of JNK1 and JNK2 correlate with the phosphorylated levels of these proteins such that the levels of non-phosphorylated JNK1 in cells decreases as the level of the phosphorylated form increases **(Fig 6A).**

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Inhibition of JNK in tunicamycin-treated HCT116 cells caused attenuation in p21CİP1 levels **(Fig 6B)**. Tunicamycin-induced p21CİP levels decreased to almost non-existence with the inhibition of JNK by SP600125. This response was not obs erved in early time periods (3 h), however at 12 h, p21 protein is almost completely degraded. This result implies that JNK activity plays a role in the stabilization of p21CİP1. Next, we want ed to determine if p21CİP1 expression contributes to the cell cy cle arrest observed with tunicamycin -treatment by evaluating the cell cycle phase distribution of SP600125 and tunicamycin -treated cells (TM treatment and JNK inhibition) compared to only tunicamycin -treated HCT116 cells.

A-

C	DMSO C	1h	3h	6h	12h	24h

pJNK
pJNK2 – 54 kDa
pJNK1 – 46 kDa

β-actin

C	DMSO C	0.5h	1h	3h	6h	12h	24h	48h

JNK
JNK2 – 54 kDa
JNK1 – 46 kDa

B-

C	TM				TM+SB600125		
	3h	12h	24h	48h	3h	12h	24h

p21CİP1

β-actin
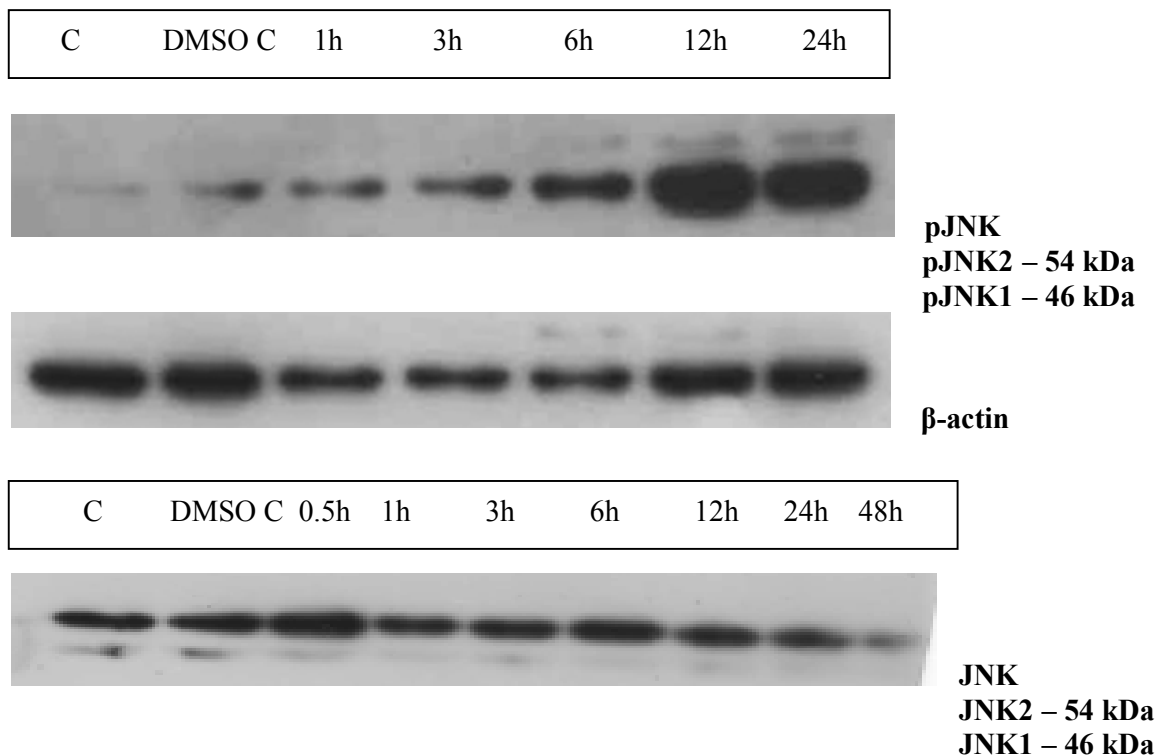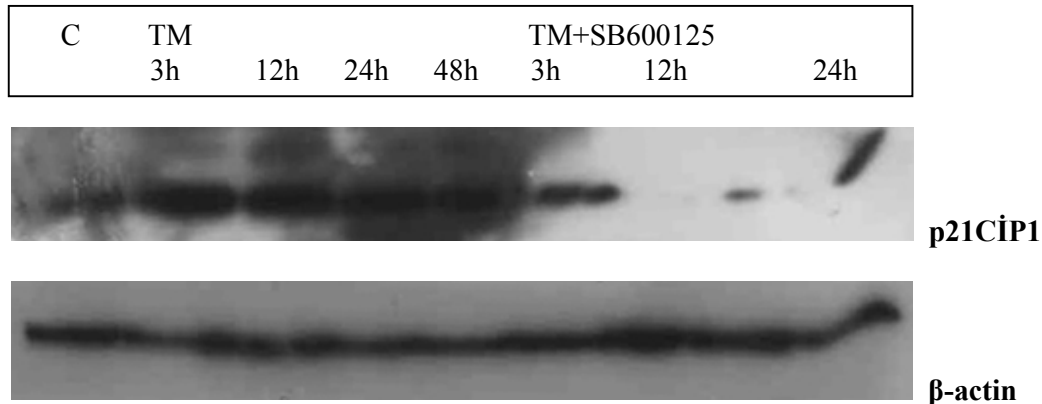
Figure 4.6A. JNK expression profile in HCT116 cells.

wt HCT116 cells were treated with TM (1 μg/ml) for various periods (0, DMSO C, 1, 3, 6, 12, 24 h) and 30 μg of total protein isolates were run on 10% SDS -PAGE gel, and were subjected to Western blot analysis for pJNK, JNK and β -actin as loading control.

Figure 4.6B. JNK inhibition attenuates p21CIP1 levels in TM -treated HCT116 cells.

p21CİP1 protein levels with JNK inhibition via SP600125 and β-actin loading control.

4.1.7- Effect of JNK, ERK and p38 Inhibition on Cell Cycle in Tunicamycin -treated HCT116: Consistent with the p21CİP1 loss due to JNK inactivity during th e UPR, G1 phase arrest induced by tunicamycin was bypassed in SP600125 and tunicamycin -treated HCT116 cells. It has been shown that JNK1 and p38 is able to stabilize p21CİP1 in TGF -Beta treated HD3 cells [165]. Our results indicate that JNK plays a role in the G1 arrest induced by tunicamycin. Tunicamycin treatment shifts the distribution of cell cycle phase towards G1. JNK inhibition in these cells clearly shifts the distribution towards G2.

Contrary to the findings of Stephen et al. on the stabilizing effect of p38 on p21 by phosphorylation (causing G1 arrest) in TGF -β treated HD3 cells, p38 inhibition by SB203580 did not change the phase distribution of tunicamycin -treated HCT116 cells. ERK inhibition by PD98059 led to a similar phenomenon as p38 inhibition **(Figure 7A).**

	% G1	% S	% G2/M
Control	46,1	16,9	37
TM (12 h)	58,2	11,4	30,4
SB600125 + TM (12 h)	38,6	11,2	50,2
PD98059 + TM (12 h)	63,5	7,5	29
SB203580 + TM (12 h)	66,1	5,9	25,7
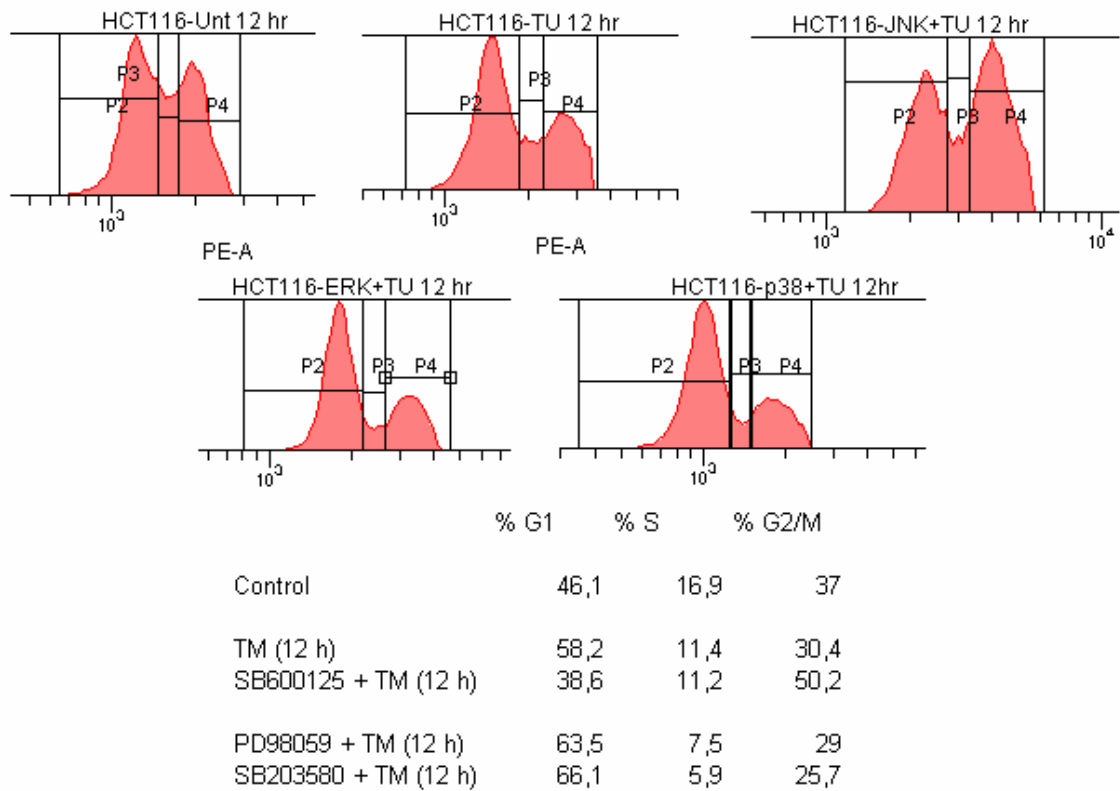
Figure 4.7. JNK, but not ERK or p38 inhibition leads cells bypass the G1 arrest induced by TM.

100,000 HCT116 cells were seeded on 12-well plates and incubated with TM for 12 h. Cells were treated with either the JNK inhibitor SB600125, p38 inhibitor SB203580, or ERK inhibitor PD98059 for 1 h prior to TM treatment. Cells were collected and subjected to PI dye for cell cycle analysis by FACS. TM-treated cells were arrested at the G1 phase of the cell cycle. SB600125 pre-treatment caused a shift in the phase distribution of TM-treated cells towards the G2 phase. P38 and ERK inhibition did not cause any shift in the distribution. JNK activity appears to play a role in the G1 phase arrest during the UPR induced by tunicamycin, possibly through stabilizing p21CİP1.

4.1.8 - Effect of Tunicamycin on Cell Death via Apoptosis and the Contribution of Caspases: The UPR activated to bring back ER homeostasis is fundamentally a cytoprotective response, in which the elements of the UPR operate in a sequential manner [20]. The first and foremost arm of the response is to decrease the ER's protein load by stopping global translation. What ensues is the increase in ER's folding capacity and the degradation of misfolded proteins.

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However, if the UPR is unable to compensate for the damage the stress exercises on the cell or when the UPR is excessive or prolonged, apoptotic mechanisms are in charge. Processing of caspase-2, -3, -4, -7, -8, -9 and -12 (in rodents) has been observed in ER stress-induced apoptosis [25].

z-VAD-fmk is a pan-caspase inhibitor that irreversibly binds to the catalytic site of caspases and inhibits the induction of apoptosis. Co-incubation of tunicamycin-treated (1μg/ml) HCT116 cells with z-VAD-fmk at 20 μM decreased tunicamycin-induced apoptosis from 22% to 16% at 24 h assayed with FACS analysis of Annexin V-binding. **(Fig 8A)**
Annexin-V binding is specific for apoptosis quantification. Apoptosis induction brings about the exposure of the phosphatidylserine (PS) residues on the cell membrane for recognition for phagocytosis. Annexin-V binds to the flipped phosphatidylserines (PS) on the cell membrane. The intensity of the signal from a single cell measured by FACS quantifies the binding of FITC-conjugated Annexin-V to the flipped PS residues. Histogram shows cell count vs. intensity of FITC radiation.

Effect of caspase inhibition on tunicamycin- treated cells was also quantified by the Tryphan blue exclusion method **(Fig 8B)** and a similar result was obtained for 15 and 27 h of treatment.
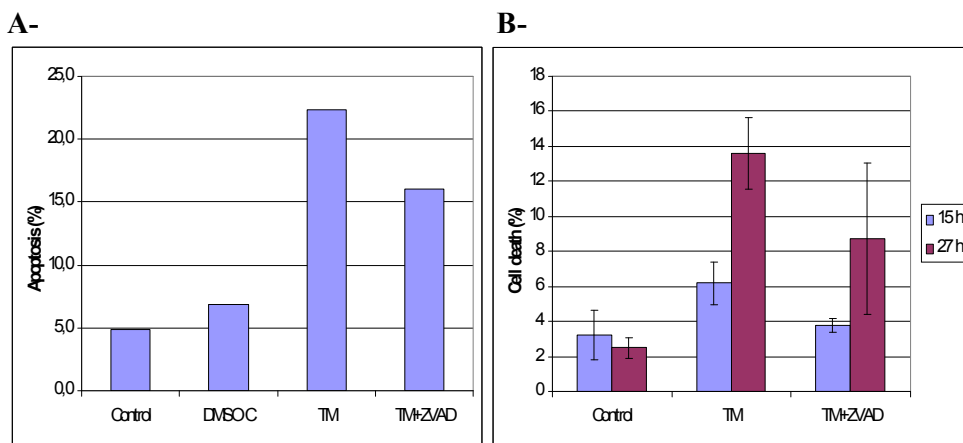
Figure 4.8. Resistance of HCT116 cells to TM-induced death upon caspase inhibition.

A, HCT116 cells were treated with the general caspase inhibitor z-VAD-fmk (20 μM) for 1 h followed by TM treatment (1ug/ml) for another 24 h. Apoptosis was quantified by FACS analysis of Annexin-V binding. **B,** HCT116 cells were treated with the general caspase inhibitor z-VAD-fmk (20 μM) for 1 h followed by TM treatment (1 μg/ml) for 15 or 27 h. Cell death was assayed by the Tryphan blue exclusion method by taking the ratio of blue cells to all counted cells. Amount of death in co-treated cells decreased.

4.1.9- Caspase Activation and PARP Cleavage Upon Tunicamycin-Treatment: Poly ADP ribose polymerase (PARP) is a substrate for caspases and it is used as a marker for caspase induction and apoptosis. PARP cleavage has been reported to be catalyzed by caspase-3 (sakvesen dixit 1997). Caspase-3 is an effector caspase downstream of the apoptosome and caspase-9 in the mitochondrial apoptosis pathway. Caspase-3 processing is observed after 24 h and PARP cleavage takes place after 48 h of tunicamycin treatment in HCT116 cells **(Fig 9A).** An interesting result is that PARP cleavage still takes place when pan-caspase processing is inhibited by the use of z-VAD-fmk. Western blotting for caspase-3 activation for cells treated with z-VAD-fmk reveals that caspase-3 processing is indeed abolished in these cells but PARP cleavage is not affected. WB for different caspases would reveal if PARP cleavage in z-VAD-fmk treated cells is dependent or independent of caspase activity.

Caspase-8 is known to mediate signal transduction downstream of death receptors located at the plasma membrane. Caspase-8 is known to process the BH3-only protein Bid, which causes Bid translocation to the mitochondrial membrane triggering cytochrome c release. Processes like this link the external apoptosis pathway with the mitochondrial pathway. Jimbo et al have shown that caspase-8 activation is indispensible for cytochrome c release and

caspase-9 activation during ER stress in P19 embryonal carcinoma cells. In our setting, we have observed caspase-8 activation **(Fig 9B)** along with caspase-3 processing and PARP cleavage upon tunicamycin treatment.

A-

PARP (full-length and cleaved)

Cleaved Casp-3

B-

Full-length Casp-8

B-actin
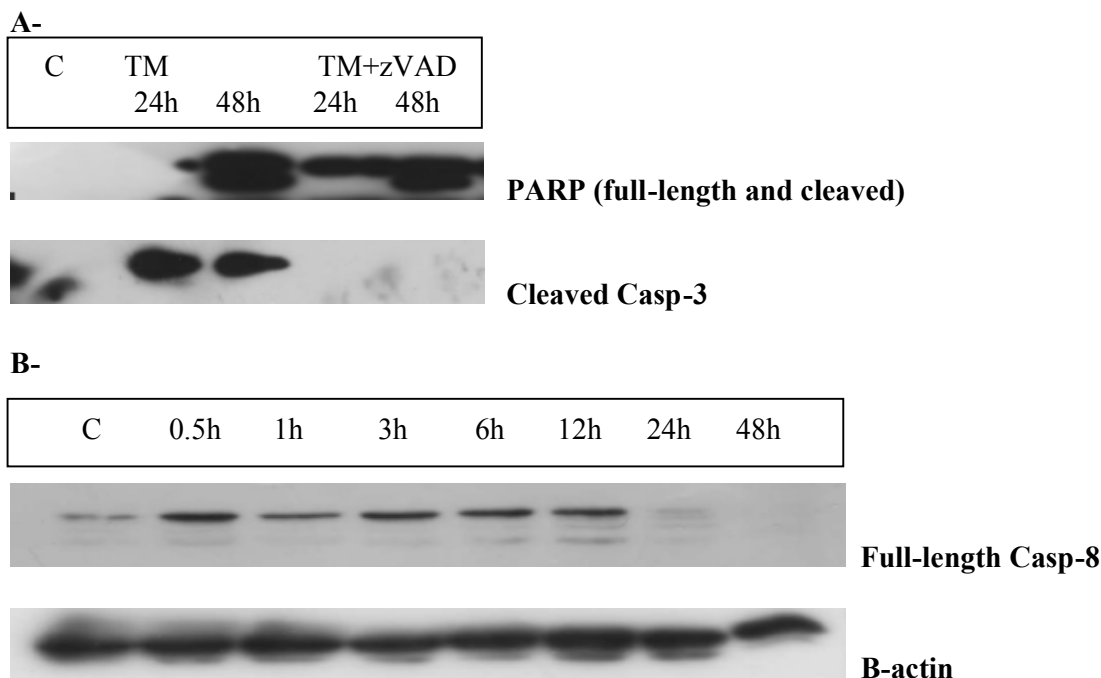
Figure 4.9A. TM induces Caspase-3 activation and PARP cleavage.

wt HCT116 cells were treated with TM (1 µg/ml) or TM (1 µg/ml) and z-VAD-fmk for various periods (0h, 24h, 48h) and 30 µg of total protein isolates were run on 10% SDS-PAGE gel, and were subjected to Western blot analysis for PARP cleavage, caspase-3 processing and β-actin as loading control. Caspase-3 processing is observed after 24 h treatment with TM. PARP cleavage takes place after 48 h. Surprisingly PARP cleavage is observed in z-VAD-fmk treated cells treated that don't activate caspase-3.

Figure 4.9B. TM induces Caspase-8 Activation.

wt HCT116 cells were treated with TM (1 µg/ml) for various periods (0, 0.5, 1, 3, 6, 12, 24, 48h) and 30 µg of total protein isolates were run on 10% SDS-PAGE gel, and were subjected to Western blot analysis for full-length caspase-8 and β-actin as loading control. Caspase-8 processing takes place in TM-treated cells after 24 h.

4.1.10 - JNK Sensitizes HCT116 Cells to Tunicamcyin-treatment after 24h: Hatai et al have reported that JNK activation induces cytochrome c re lease. JNK has also been reported to phosphorylate and inactivate the proapoptotic Bcl-2 [108]. JNK phosphorylates the prosurvival Bcl-2 it inactive. It can phosphorylate BAD to inhibit apoptosis. In contrast to its

proapoptotic function, inhibition of JNK by SP600125 have been reported to decrease the viability of some gastrointestinal cancer cell lines and induced apoptosis via the activation of caspase-8, caspase-3 and PARP cleavage [104]. The severity of the death response correlated with the level of active JNK in control cells.

JNK inactivation sensitized HCT116 cells to tunicamycin after 24 h of treatment by almost 2 - fold compared to tunicamycin-treated cells **(Fig 10).** In our case, JNK plays a prosurvival role in ER stress-related death since its inactivation increases the rate of apoptosis. In contrast, ERK and p38 inactivation do not make a pronounced difference in the apoptotic response after 24 h of tunicamycin treatment.
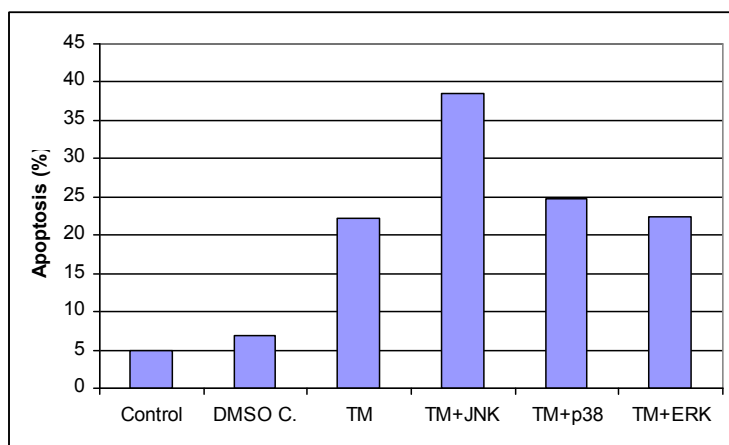
Figure 4.10. JNK inactivation sensitizes cells to ER stress after 24 h.

Cells were incubated without (Control) or with 1 ug/ml TM for 24 h in the presence or absence of either JNK, p38 or ERK inhibitors. Apoptosis was assayed by the Annexin V - FITC binding assay using FACS. JNK inhibition significantly increased the percentage of apoptosis in tunicamycin-treated HCT116 cells after 24 h. p38 and ERK inhibition did not have a significant effect on apoptosis.

4.1.11 – CHOP Levels Upon JNK Inhibition: It has been reported that overexpression of the highly induced protein CHOP during ER stress causes apoptosis. The transcription factor CHOP is known to be induced by all three arms of the UPR. **Fig 11** shows the levels of CHOP protein upon TM treatment and JNK inhibition. JNK inhibition in TM-treated HCT116 cells decreasesd the levels of CHOP after 12 hr of treatment. This result implies that JNK activity has a stabilizing effect on CHOP and that CHOP is not responsible for increased apoptosis observed with JNK inhibition in TM -treated HCT116 cells.

| C | TM | | | | TM+SB600125 | | |
| | 3h | 12h | 24h | 48h | 3h | 12h | 2 4h |
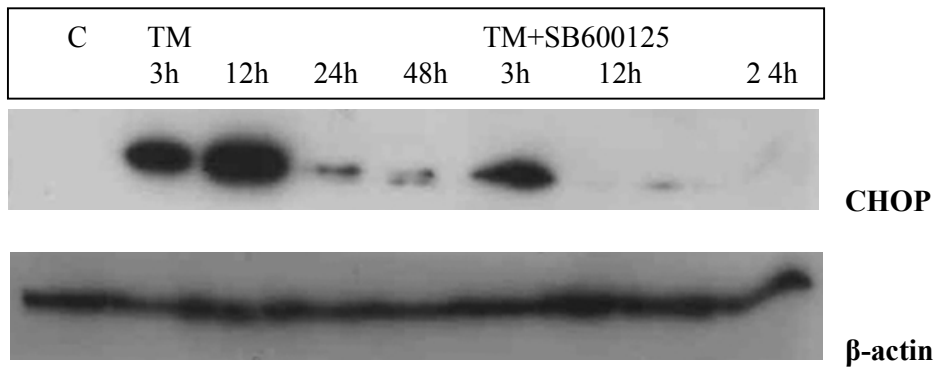
CHOP

β-actin

Figure 4.11. JNK inhibition attenuates CHOP levels in TM -treated HCT116 cells.
CHOP protein levels with JNK inhibition via SP600125 and β-actin loading control

4.1.12 – Effect of p38 and ERK Inhibition on Cell Survival : Phosphorylation levels of ERK1/2 and p38 in control and tunicamycin -treated HCT116 cells are shown in **Fig 12**. Phosphorylated ERK1/2 is constitutively active in control cells and levels of activation were not increased by treatment with tunicamycin. Levels of active p38 decrease with treatment. The prosurvival effect of pERK is observed at 40 h of treatment by the increase in cell death when the activation of ERK1/2 is inhibited by the molecule SB203580.

p38 phosphorylation status and contribution to cell death is tougher to evaluate. Active p38 levels decrease with treatment and p38 inhibition causes an increase in cell death at 40 h.

A-

	C	0.5h	1h	3h	6h	12h	24h	48h

p-ERK1/2

β-actin

	C DMSO C	0.5h	1h	3h	6h	12h

p-p38

β-actin
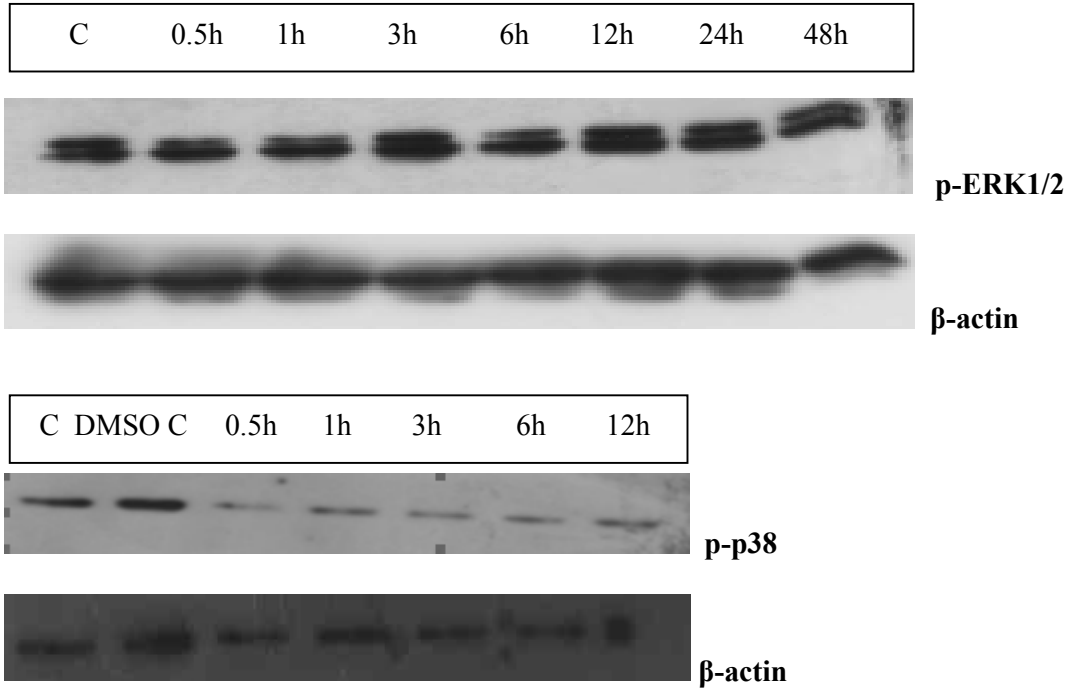
B-
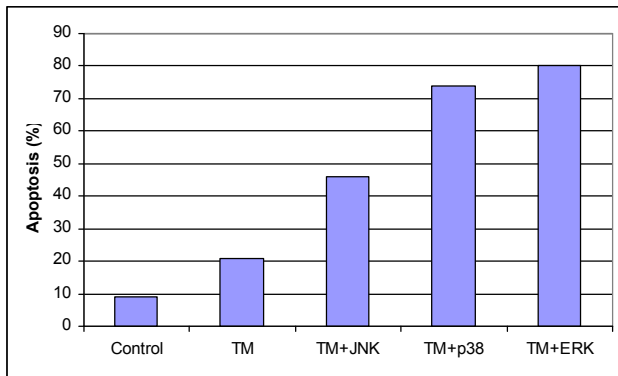
Figure 4.12A. Levels of ERK and p38 phosphorylation.

wt HCT116 cells were treated with TM (1 μg/ml) for various periods and 30 μg of t otal protein isolates were run on 10% SDS-PAGE gel, and were subjected to Western blot analysis for pERK, p -p38 detection and B-actin as loading control.

Figure 4.12B. Inactivation of p38 and ERK sensitizes HCT116 cells to TM after 40 h of treatment.

FACS analysis of apoptosis in TM -treated HCT116 cells incubated without (control) or with tunicamycin (TM) in the presence or absence of MAPK inhibitors (JNK, p38 or ERK) for 40 h. Active p38 and ERK have a greater and death preventing effect on apoptosis at 40 h than 24 h of treatment.

4.1.13 – Role of MAPK on HCT116 Cell Survival: Control HCT116 cells were treated with MAPK inhibitors and induction of apoptosis was quantified by FACS analysis of Annexin -V binding. All three MAPK are active constitutively in non-treated HCT116 cells (**Figs 8 and 13**). Inactivating JNK in non-treated cells leads to an apoptotic response (**Fig 13**). The JNK pathway seems to be crucial for the survival of HCT116 colon carcinoma cells. p38 and ERK activity do not seem to contribut e to cell survival in these cells since their inhibition does not elicit an apoptotic response.

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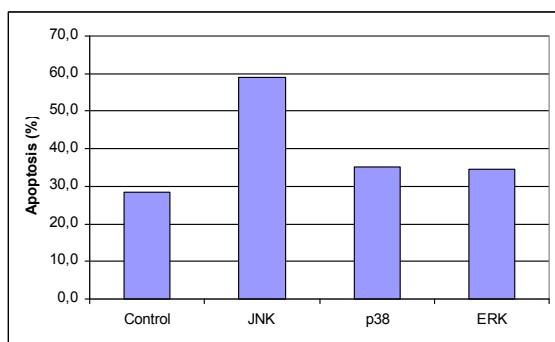
Figure 4.13. Role of MAPK on HCT116 cell survival.

wt HCT116 cells were incubated without (control) or with 20 μM MAPK inhibitors of JNK, p38 or ERK for 24 h. Inhibitors were given 1 h prior to TM -treatment. JNK inhibition was significant in increasing the percentage of apoptotic cells.

4.2 Discussion

The ER is responsible for the translation, maturation, modification and delivery of secretory proteins. Maintenance of ER homeostasis is crucial for a healthy operating cell. ER homeostasis can be lost due to many factors related to the proper working conditions and undertakings of the ER. Loss of homeostasis at the ER results in ER stress and the act ivation of the unfolded protein response (UPR) to alleviate this stress [15]. The UPR induces cell cycle arrest at the G1 phase. This is mostly due to global translational attenuation during the UPR mainly as a result of eIF2α phosphorylation by the activated PERK kinase [162].

Prolonged ER stress eventually leads cells to apoptosis (typically) through multiple potentially participating processes, such as the activation of caspase -12 in rodents [83], activity of MAP kinases [92], induction of CHOP protein [123], release of Ca^{2+} from the ER [27], alterations in Bcl-2 family proteins, such as the phosphorylation of proapoptotic BH3 - onlys [130, 131] etc. Signals from the ER are merged with the intrinsic apoptosis pathway by

the activity of the Bcl-2 family members. Cytochrome c release and caspase-9 activation related to mitochondrial membrane polarization (MOMP) is observed in ER stress -related cell death [80]. It has been reported that caspase-8 plays a role in merging the death signals with the mitochondrial apoptosis pathway in ER stress -related apoptosis [171].

Here, we have assessed the role of MAPK in ER stressed cancer cells related to cell proliferation and cell death with an emphasis in JNK activity. Contributions of p38 and ERK to the condition of the ER-stressed cell were mainly evaluated in comparison to JNK activity.

In physiological conditions, the UPR is activated in response to acute or persistent stress. Cancer cells that are subjected to hypoxia have been reported to adapt to persistent ER stress that may result in resistance to chemotherapeutics. Here, we have used the HCT116 colon carcinoma cell line and mimicked the UPR by using tunicamycin as an ER stress inducer at a concentration that resulted in cells' demise in a two day period (48h). The activation of the cytoprotective UPR comprised the primary response that lasted for one day, after which cells started to die at least partially by apoptosis.

Fig 1 shows the cell death response of HCT116 cells upon tunicamycin treatment with increasing concentrations and at different time periods (12, 24, 48 h) assessed by the MTT assay, which quantifies cell viability by cell metabolic activity. The death response is not observed after 12 h treatment. The death response observed after 24 and 48 h treatments did not increase with increasing the drug concentration, whereas death related to DMSO concentration did. A supposition could follow with the observation that increasing the drug concentration does not significantly affect cell death that the availability of the three ER stress-sensing transmembrane proteins, PERK, ATF6 and IRE1 may be a liming factor in the stress response behavior of cells, and that the concentration of 1 μg/ml tunica mycin activates most of the available sensor proteins at the ER membrane for signaling. We chose the concentration of 1 μg/ml for further experimentation for the reason that DMSO at that concentration gave the lowest death response and cell death response was expanded to a two day period.

Much of the knowledge about the UPR, its activation and regulation, comes from cultured cells treated with ER stress-inducing agents, such as tunicamycin. It should be noted that this kind of experimentation bares a short fall in its nature that it fails to imitate the UPR under

milder and persistent assault, which is often the case in the physiological condition. There are cases, where cells, when faced with chronic stress activate the UPR in an adaptive form and escape death in a scale ranging from days to years. Treatment with these agents severely assaults the cells under investigation and eventually activates the death mechanism linked with the UPR. In vitro experimentation, therefore, may not be the equivalent of the physiological UPR or more precisely, the problem with this methodology is that it circumvents the question of how cells escape death and adapt to ER stress [15].

CHOP protein is known to be induced by all three arms of the UPR [43, 172, 173]. Upon ER stress, the transcriptional induction of CHOP is regulated by four *cis*-acting elements, AARE1, AARE2, ERSE1 and ERSE2 [174]. Under ER stress, the ER-resident chaperone BiP preferentially binds to misfolded/unfolded proteins at the ER lumen rendering active the three ER transmembrane stress transducers PERK, ATF6 and IRE1 [28, 175]. Activated PERK phosphorylates eIF2α, ceasing global protein translation [2] and resulting in the upregulation of the transcription factor ATF4, that contains an upstream open reading frame (uORF) [35]. One of the targets of ATF4 is CHOP. ATF6 is transported to the Golgi where it is cleaved by S1P and S2P proteases to become an active transcription factor that activates CHOP [40]. Activated IRE1 cleaves an intron of the XBP-1 mRNA available in the cytoplasm, making it a mature mRNA with an open reading frame for transcription. XBP-1 transcription is also regulated by ATF6 [48]. ATF4 binds to the AARE1 and AARE2 motifs on the *chop* promoter. Both ATF6 and XBP-1 bind to the ERSE motifs with the transcription factor NF-Y [174].

Figure 2 shows the elevated CHOP protein levels upon tunicamycin treatment that lasts for the first 24 h. We have used the induction of CHOP as a marker of UPR induced by tunicamycin that cells are undergoing the UPR. CHOP protein is not expressed in control cells because CHOP is expressed under the condition that eIF2α is phosphorylated. The decrease in CHOP expression after 24 h may be due to the activation of eIF2α. A western blot for phosphorylated eIF2α may clarify the expression profile of CHOP during the UPR. As to the death promoting nature of CHOP, overexpression of CHOP induces apoptosis that can be inhibited by the prosurvival apoptotic molecule Bcl-2 [123]. However, perk-/- cells that are extremely sensitive to ER stress does not require CHOP for apoptosis [33]. Downstream of CHOP-induced apoptosis is not clear [81]. CHOP is known to be modified

post-transcriptionally. p38 MAP kinase increases CHOP transcriptional activity by phosphorylating it on Ser78 and Ser81 [176].

In this study, we evaluated the role of JNK in UPR conditions because JNK is a stress-activated kinase that has been reported to be involved in cell proliferation and apoptosis in many types of death-inducing stimuli. There are three forms of JNK, JNK1-3. JNK1 and JNK2 are expressed ubiquitously and JNK3 is specific to the brain, testes and heart [93]. JNK is activated by various kinds of environmental stress, such as osmotic stress, redox stress, radiation and treatment with cytokines, such as TNF, IL-1 [93].

The role of JNK in apoptosis seems to be cell type and stimulus-specific and that JNK1 and JNK2 have differential roles in contributing to cell death or proliferation. JNK1 and JNK2 deficient mice show different patterns of gene expression [97].

A proapoptotic role for JNK has been described in vinblastine, doxorubicin and etoposide-induced apoptosis [112]. In contrast, Vivo et al have shown that JNK inhibited TRAIL-induced apoptosis [177].

The contradictory observations for the effect of JNK activation on cell condition have been attributed to its large number of substrates and their temporal availability [96].

Constitutive JNK basal activity has been reported in some cancers. Wang et al have examined cancer and normal human breast tissues and found that the expression of JNK2 was markedly increased in cancerous tissues [102]. In our case, control HCT116 cells express active JNK1 but not JNK2 without any external stimulus (Fig 6). The constitutive biologic function of basally active JNK is thought to promote cell survival and growth [101]. There are studies suggesting that JNKs might have an oncogenic role. In cancer cells, an 'oncokinase' would transduce signals leading to cell survival and proliferation. We have observed a similar phenomenon with HCT116 cells, that when JNKs are inhibited by the specific JNK inhibitor SP600125, apoptosis was induced (Fig 13). The inhibition of the other two MAPKs ERK and p38 did not induce apoptosis. The inhibition of JNKs has been shown to cause growth arrest or apoptosis in some tumor cells [103]. Xia et al investigated the levels of active JNK in various gastrointestinal cancer lines and found that the apoptotic effect of JNK inhibition in untreated cancer cells is correlated with the strength of basal JNK activity,

in their case JNK2. The oncogenic activity of JNK may be related to the transformation of the cells because a contrary report is that doubly knocking out jnk1-/-jnk2-/- in fibroblasts cause an increase in the number and growth of Ras-induced tumor nodules.

ER stress induces cell cycle arrest at the G1 p hase. Cyclin-dependent kinase (CDK)-mediated inactivation of the retinoblastoma tumor suppressor protein (pRB) is required for cells to undergo the G1 to S phase transition. CDK4 and CDK6 are activated during mid -G1 and their activity depends on proteins of the cyclin D family. Active CKD4 and CKD6 phosphorylate pRB rendering it inactive. This promotes the expression of genes required for the G1 to S transition, such as cyclin E that is induced in late G1. Cyclin E binds and activates CDK2, cell cycle continues.

Tunicamycin-induced-cell cycle arrest is mainly due to the phosphorylation of the eIF2α by PERK kinase. eIF2α phosphorylation induces an arrest in global translation, which effects cyclin D1 translation and protein level as well. Cyclin D1 is required for passing the early checkpoint in the G1 to S phase transition. Cyclin D1 phosphorylates and activates the cyclin-dependent kinase CDK4 and contributes to the progression of cell cycle. Cyclin D1 has a short half-life and is depleted quickly in the case of t ranslational attenuation.

CDKs are inhibited by CDK inhibitor proteins (CKIs) including p21CİP1 and p27KİP1. Cell cycle arrest can be caused the degradation of cyclin subunits, or to the induction of CDKIs.

Even though the CKIs break the cyclin A - CDK2 and cyclin E - CDK2 complexes, they positively regulate the cyclin D - CDK4/6 complexes [153] which are essential during the early time points of the G1 to S transition. The UPR induces cell cycle arrest by blocking the translation of cyclin D1 mRNA during the global translational attenuation caused by the phosphorylation of the elongation factor eIF2 α via PERK kinase activity [162]. In this case, CİP/KİP proteins are free to bind the cyclin E – CDK2 and cyclin A – CDK2 complexes to inactivate them. This process contributes to the G1 arrest observed with tunicamycin treatment.

p21CİP1 is a global inhibitor of cell cycle progression at the G1 to S transition. p21CİP1 has a short-half life of about 20 to 60 minutes in most cells. It is degraded by t he

ubiquitin/proteasome pathway [158]. p21CİP1 can be regulated posttranscriptionally to stabilize it [157].

We have shown that p21CİP1 levels increase with tunicamycin -treatment (Fig 5). We have also observed the G1 arrest induced by tunicamycin (Fig 5). We speculate that this phase arrest is partly due to the increase in p21CİP1 levels. JNK inhibition via SP600125 t reatment decreased the levels of p21CİP levels in tunicamycin -treated HCT116 cells (Fig 6). This decrease was effective after the 3rd hour of treatment until the 24th hour. This observation correlated with the cell cycle progression of these cells. Cell cy cle analysis by PI dye assayed by FACS revealed that tunicamycin-treated cells were arrested at the G1 phase of the cell cycle, whereas JNK inhibition led cells bypass this arrest and shifted the phase distribution towards the G2 phase (Fig 7). At 12 hours, JNK inhibited tunicamycin-treated cells continued to proliferate (observed by microscopy as well) in contrast to only tunicamycin - treated cells. Inhibition of the two MAPK p38 and ERK did not have an effect (Fig 7).

The increase in p21CİP1 protein levels is less likely due to a translational or transcriptional increase since protein translation does not take place during the UPR. Absence of p21CİP in cells treated with the JNK inhibitor SP600125 suggests that the post -translational stabilization of p21CİP1 includes JNK's kinase activity. JNK and p38 have been reported to phosphorylate and stabilize p21CİP1 [165]. We can speculate that the absence of p21CIP1 in the case of JNK inhibition facilitates the G1 to S transition in these cells. The reappearance of p21CIP1 may be related to the resume of protein translation. Attenuation in translation that takes place promptly as a part of the UPR is nevertheless transient. Western blot for phosphorylated eIF2α may clarify the situation. It is also important to note that the appearance of p21CIP1 in JNK-inhibited tunicamycin-treated HCT116 cells correlates with the initiation of apoptosis in these cells.

The mechanisms that cause the switch to apoptosis from the survival response during the UPR are not clear. The dominating facto rs are thought to differ for different cell types. JNK activation by the ER stress transducer IRE1 through TRAF2 and ASK1, induction of CHOP, deregulation of Bcl-2 and IAPs (inhibitor of apoptosis), upregulation of proapoptotic BH3 - onlys have been suggested as mediators of apoptosis in the case of UPR. Mitochondrial (intrinsic) apoptosis pathway is involved in ER stress -induced apoptosis [80].

Tunicamycin-treated HCT116 cells underwent apoptosis after 24 h of treatm ent (Fig 8). Cell death was not observed in the first 12 hours of treatment, assessed by MTT assay and the Tryphan blue exclusion method that measure cell viability through cell metabolic activity and cell death, respectively. Annexin V-FITC binding assay evaluated by FACS did not suggest apoptotic death in the first 12 hours. This is sensible considering the fact that UPR is activated as a cytoprotective mechanism in cells subjected to ER stress, and that if the UPR is not overcome, cells start to die.

We evaluated the dependence on caspases in tunicamycin -induced cell death in HCT116 cells using two methods. We have used z-VAD-fmk, a pan-caspase inhibitor and quantified apoptosis by FACS and cell death by the Trypan blue exclusion method. Apoptosis decre ased from 22% to 16% in tunicamycin-treated cells. A similar decrease was observed with the Tryphan blue method. The reason for a non -complete attenuation may be that ER -stress induced apoptosis in HCT116 cells may involve caspases that are not inactivated by z-VAD-fmk or that apoptosis related to ER stress in these cells are only partially caspase -dependent.

Next, we evaluated cell death in the case of JNK, ERK and p38 inhibition. We showed that the inhibition of both JNKs, JNK1 and JNK2 by SP600125 enhan ces the rate of ER-stress induced apoptosis HCT116 colon carcinoma cell line by about 2 -fold after 24 hours with no further increase after 24 h (Figs. 10 and 12). Inhibition of p38 or ERK MAPKs does not have an effect in the first 24 h but their inhibition is effective in increasing apoptosis after 40 h of treatment. Phosphorylated ERK is present in control cells and tunicamycin treatment does not affect the levels. Phosphorylated p38 levels decrease with tunicamycin treatment, the effect of which is tough to evaluate.

Next, we wanted to figure out what causes the increased apoptotic response when JNK is inhibited in TM-treated cells. We inspected the level of the transcription factor CHOP since CHOP is highly induced during ER stress, and that its overexp ression causes growth arrest and apoptosis [120, 121]. We have found that CHOP is not responsible for increased apoptosis in JNK-inhibited cells. Figure 11 shows that CHOP is almost non -existent in TM-treated and JNK-inhibited HCT116 cells at 12 h of treatment. Therefore, we conclude that the increase in death related to JNK inhibition is not rela ted to elevated levels of CHOP. The result also suggests that active JNK has a role in stabilizing the CHOP protein.

The proliferative effect of JNK inhibition in the first 12 hours of treatment, and death inducing effect of JNK inhibition after 24 hours may seem contradictory. However, both responses may be thought to define a prosurvival role for JNK activity. In t he case of promoting G1 arrest, JNK plays a cytoprotective role through the conservation of metabolic energy. One suggestion for differential roles of JNK pertaining to the different phases of the UPR is that JNK's role differs with the temporal availability of its substrates. In the case of ER stress, JNK might be serving different roles in re lation to the status of eIF2α activity which determine the on/off status of global translation. Data for eIF2α phosphorylation could come in handy in evaluating the differential effects of JNK. p38 and ERK show differential effects on cell death in similar ways during the course of ER stress response.

4.3 Conclusion

As a summary, in our experimental model we have found that JNK plays a prosurvival role during the different phases of ER stress. During the survival response, JNK activity promotes the G1 cell cycle arrest induced by tunicamycin. This arrest may be thought as a survival mechanism since termination of division conserves energy. Later in the response, inhibition of JNK increases the apoptotic response by about 2 -fold after 24 h of treatment. p 38 and ERK inhibition does not have an effect either on the cell cycle distribution or death profile at 24 h of treatment. The apoptosis -inducing effect of p38 and ERK inhibition can be observed after 40 h of treatment.

The elucidation of the prosurvival factors pertaining to ER stress management of cells is important in finding new strategies for cancer therapy. Cancerous tissue that receives insufficient oxygen and nutrition undergoes ER stress. In many conditions, the UPR is an adaptation to persistent ER stress. It has been reported that in this case cells are more resistant to cancer therapy. Administration of drugs aimed at the prosurvival elements of the UPR may render therapy more effective.

Apart from resulting in apoptosis, ER stress induces aut ophagy as well. The role of autophagy in cell death is a debated issue. It is thought that autophagy serves a cytoprotective role early in its induction that it aids the mechanism of eliminating misfolded proteins. There are cases in experimental condition s, where autophagy results in cell death. It would be interesting to evaluate the role of autophagy in ER -stress induced death. Inhibition of the

PI3K by its inhibitor LY294002 increases apoptosis in these cells (data not shown) and PARP cleavage is observed more rapidly. LY294002 is also known to inhibit autophagy by inhibiting the formation of the autophagosome. We would like to evaluate if the contribution of autophagosome formation promotes survival or death in the ER -stressed cells and that if this is in any way related to JNK kinase activity.

p21CİP1 translation is achieved through p53 -dependent and independent mechanisms. As a future perspective, the dependence of p21 induction on p53 could be evaluated for HCT116 cells that lack the p53 gene. PCR analysis of p21CİP1 mRNA levels and Western blotting for eIF2α phosphorylation may serve as evidence for JNK's role in p21C İP1 stabilization.

It would be interesting to investigate if the increased induction of apoptosis related to JNK inactivity is related to increased caspase activation. We found that PARP cleavage is not inhibited in cells treated with the pan -caspase inhibitor z-VAD-fmk. We could use specific caspase inhibitors and western blotting to eliminate the caspases that do not take part in the cleavage of PARP during ER stress.
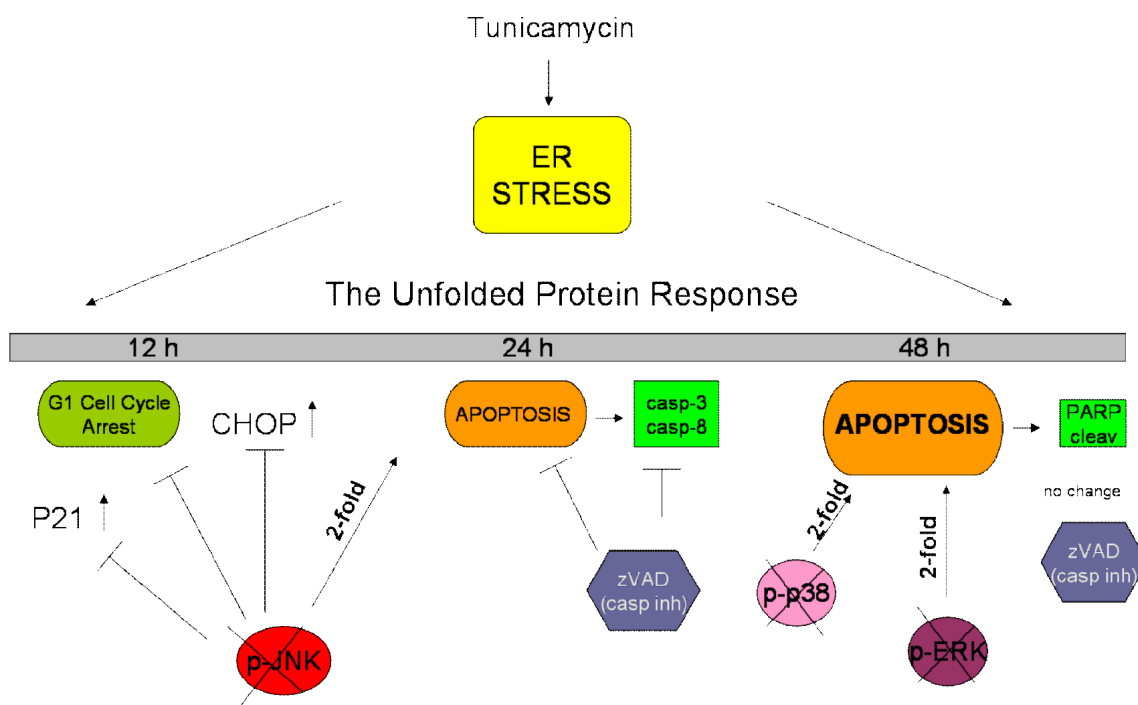

Figure 4.14 Suggested Mechanism for the Unfolded Protein Response in HCT116 cells related to the MAPKs JNK, p38, ERK, and caspase activity, p21 and CHOP protein levels.

Appendix A

CHEMICALS (in alphabetical order)

Name of Chemical	Supplier	Catalog Number
Acrylamide/Bis-acrylamide	Sigma, Germany	A3699
Ammonium persulfate	Sigma, Germany	A3678
Annexin V-FITC	Alexis Biochemicals	ALX-209-250-T100
Antibiotic solution	Sigma, Germany	PP3539
Anti β-actin Ab	Cell Signal Tech, USA	4967
Anti-JNK Ab	Cell Signal Tech, USA	9252
Anti p-JNK Ab	Cell Signal Tech, USA	4671
Anti-ERK1/2 Ab	Cell Signal Tech, USA	9102
Anti-p-ERK1/2 Ab	Cell Signal Tech, USA	4377
Anti-p38 Ab	Cell Signal Tech, USA	9212
Anti-p-p38 Ab	Cell Signal Tech, USA	9215
Anti p21	Cell Signal Tech, USA	2946
Anti-cleaved caspase 3 Ab	Cell Signal Tech, USA	9661
Anti-caspase 8 Ab	Cell Signal Tech, USA	
Anti-rabbit IgG HRP linked	Cell Signal Tech, USA	7074
Anti-mouse IgG HRP linked	Amersham Biosciences, UK	RPN4201
Anti-CHOP	Santa Cruz Biotech.	
DMSO	Sigma, Germany	D2650
Ethanol	Riedel-de Haen, Germany	32221
Feotal Bovine Serum	Sigma, Germany	F2442
Glycine	Amnesa, USA	0167
HCl	Merck, Germany	100314
Hyperfilm ECL	Amersham Biosciences, UK	RPN2103K
Hybond P-membrane (PVDF)	Amersham Biosciences, UK	RPN2020F
Isopropanol	Riedel-de Haen, Germany	24137
KCl	Fluka, Switzerland	60129
KH_2PO_4	Riedel-de Haen, Germany	04243
KOH	Riedel-de Haen, Germnay	06005
Liquid Nitrogen	Karbogaz, Turkey	
McCoy's 5A Medium	Biological Industries	01-075-1
2-Mercaptoethanol	Sigma, Germany	M370-1
Methanol	Riedel-de Haen, Germany	24229
$MgCl_2$	Sigma, Germany	M9272
Milk Diluent Concentrate	KPL, USA	50-82-00
NaCl	Riedel-de Haen Germany	13424
NaO2H2H3.3H2o	Riedel-de Haen, Germany	25022
NaOH	Merck, Germany	1006462
NaPO4H2	Riedel-de Haen Germany	04269
NP-40	Sigma, Germany	I3021
Pen/Strep Solution	Biological Industries	
PD98059	Calbiochem, USA	513000
PMSF	Sigma, Germany	P7626
Prestained Protein MW	Fermantas, Germany	#SM0441

Propidium Iodide (PI)		
Rnase A	Roche, Germany	1119915
SB2033580	Calbiochem, USA	559389
SP600125	Calbiochem, USA	420123
Sodium Dodecyl Sulphate	Sigma, Germany	L4390
TEMED	Sigma, Germany	T7029
Triton X-100	Applichem, Germany	A1388
Tris	Fluka, Switzerland	93349
Tunicamycin	Calbiochem, USA	654380
Trypan Blue Dye	Merck, Germany	
Trypsin/EDTA 1X	Merck, Germany	03-050-1
Tween-20	Merk, Germany	822184

Appendix B

Molecular Biology Kits (in alphabetical order)

Name of Kit   **Supplier**   **Catalog Number**

ECL Advanced Chemiluminescence	Amersham Biosciences, UK	RPN2135
Cell Proliferation Kit I (MTT)	Roche, Germany	1465007-001
Dc Assay	Bio-Rad	

Appendix C

Autoclave	Hirayama, Hiclave HV-110, Japan
	Certoclav, Table Top Autoclave CV-EL-12L, Austria
Balance	Sartorius, BP211D, Germany
	Sartorius, BP221S, Germany
	Sartorius, BP610, Germany
	Schimadzu, Libror EB-3200 HU, Japan
Centrifuge	Eppendorf, 5415C, Germany
	Eppendorf, 5415D, Germany
	Eppendorf, 5415R, Germany
	Kendro Lab. Prod., Heraeus Multifuge 3L, Germany
	Hitachi, Sorvall RC5C Plus, USA
	Hitachi, Sorvall Discovery 100 SE, USA
Deepfreeze	-70°C, Kendro Lab. Prod., Heraeus Hfu486 Basic, Germany
	-20°C, Bosch, Turkey
Distilled Water	Millipore, MilliQ Academic, France
Electrophoresis	Biogen Inc., USA
Ice Machine	Scotsman Inc., AF20,USA
Incubator	Memmert Modell 300, Germany
	Memmert Modell 600, Germany
Laminar Flow	Kendro Lab. Prod., Heraeus, HeraSafe HS12, Germany
Magnetic Stirrer	VELP Scientifica, ARE Heating Magnetic Stirrer, Italy
Microliter Pipette	Gilson, Pipetman, France
	Mettler Toledo Volumate, USA
	Eppendorf, Germany
Microscope	
pH Meter	WTW, pH540 GLP MultiCal, Gerany

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Power Supply	Biorad, PowerPac 300, USA
	Wealtec, Elite 300, USA
Refrigerator	4°C, Bosch, Turkey
Shakers	Forma Scientific, Orbital Shaker 4520, USA
	GFL Shaker 3011 USA
	New Brusnswick Sci., Innova 4330, USA
	C25HC Incubator shaker, New Brunswick Scientific, USA
Spectrophotometer	Schimadzu UV-1208, Japan
	Scimadzu UV-3510, Japan
Thermocycler	Eppendorf, Mastercycler Gradient, Germany
Water bath	Huber, Polystat cc1, Germany

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